IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Semple, et al.	Art Unit	: 1626
Serial No.	: 10/535,345	Examiner	: Susannah Lee Chung
Filed	: February 15, 2006	Conf. No.	: 6159

Title : TETRAZOLE DERIVATIVES AND METHODS OF TREATMENT OF METABOLIC-RELATED DISORDERS THEREOF

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

BRIEF ON APPEAL

(1) Real Parties in Interest

The real parties in interest are Arena Pharmaceuticals, Inc. and Merck & Co., Inc. by virtue of assignments recorded at reel/frame nos. 020543/0539 and 019962/0277.

(2) Related Appeals and Interferences

There are no related appeals or interferences.

(3) Status of Claims

Claims 1-64 are canceled.

Claims 65-67 are rejected.

Applicants are appealing the rejection of claims 65-67.

(4) Status of Amendments

No amendments have been submitted after the Office Action mailed on June 1, 2010, and no amendments are pending.

(5) Summary of Claimed Subject Matter

Independent claim 65 reads as follows:

65. A compound, which is 3-(1H-tetrazol-5-yl)-2,4,5,6-tetrahydro-cyclopentapyrazole, or a pharmaceutically acceptable salt, solvate or hydrate thereof.

Basis for independent claim 65 in the specification as originally filed can be found at page 29, line 2 (Table A, Compound 1); page 3, line 19; and in original claim 7. It will be recognized that independent claim 65 encompasses the tautomer, which is alternatively expressed as 3-(1H-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole, as generally described at page 13, lines 5-19 of the as-filed specification and specifically described at page 59, lines 13-14 (Example 9.1, Compound 1). The compound in independent claim 65 is also known as MK-0354. *See* Graeme Semple, et al., *3-(1H-Tetrazol-5-yl)-1,4,5,6-Cyclopentapyrazole (MK-0354): A Partial Agonist of the Nicotinic Acid Receptor, G-Protein Coupled Receptor 109a, with Antilipolytic But No Vasodilatory Activity in Mice*, 51 J. MED. CHEM. 5101, 5101-5108 (2008).

Independent claim 66 reads as follows:

> 66. A pharmaceutical composition comprising 3-(1H-tetrazol-5-yl)-2,4,5,6-tetrahydro-cyclopentapyrazole, or a pharmaceutically acceptable salt, solvate or hydrate thereof, in combination with a pharmaceutically acceptable carrier.

The compound recited in independent claim 66 is the same as in independent claim 65. Basis for independent claim 66 in the specification as originally filed can be found as described above; and at page 3, lines 20-21.

Independent claim 67 reads as follows:

> 67. A method of lowering free fatty acids in an individual comprising administering to said individual a therapeutically-effective amount of 3-(1H-tetrazol-5-yl)-2,4,5,6-tetrahydro-cyclopentapyrazole, or a pharmaceutically acceptable salt, solvate or hydrate thereof.

The compound recited in independent claim 67 is the same as in independent claims 65 and 66. Basis for independent claim 67 in the specification as originally filed can be found as described above; at page 32, lines 3-4; at page 34, lines 4-6; and at page 10, lines 14-15.

(6) Grounds of Rejection to be Reviewed on Appeal

I. Claim 67 is rejected under 35 U.S.C. § 112, ¶ 1, as being non-enabled with respect to the recited method.

II. Claims 65-67 are rejected under 35 U.S.C. § 112, ¶ 1, as being non-enabled with respect to the terms "solvate" and "hydrate".

These rejections were made in a final rejection mailed June 1, 2010. The rejections are ripe for appeal under 37 CFR § 41.31 because claims of the application have been rejected at least two times (four times in total): (a) in a non-final office action mailed June 24, 2008; (b) in a final rejection mailed March 18, 2009; (c) in a non-final office action mailed October 19, 2009; and (d) in the final rejection mailed June 1, 2010.

(7) Argument

As will be appreciated, § 112, ¶ 1 "does not require that a specification convince persons skilled in the art that the assertions therein are correct". *In re Armbruster*, 512 F.2d 676, 678, 185 U.S.P.Q. 152, 153 (C.C.P.A. 1975). Instead, the burden is on the Office "to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement". *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 369-370 (C.C.P.A. 1971). Hence, the threshold issue should not be whether Appellants' specification convinces the Examiner that the claimed methods, solvates and hydrates are enabled, but, rather, whether the Examiner has met the initial burden of providing a substantiated explanation as to why they aren't. In the instant case, the Examiner has failed to substantiate the allegation that the claims 65-67 are non-enabled.

I. <u>The method of claim 67, on appeal, is enabled.</u>

This is an enablement rejection under 35 U.S.C. §112 ¶ 1 with respect to the method of lowering free fatty acids in an individual in claim 67. The proper standard for an enablement inquiry rests on whether one skilled in the art would be able to make and use the invention without undue experimentation. *In re Wands*, 858 F.2d 731, 737, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 2008). Factors for consideration in determining whether undue experimentation is necessary to make and use the invention include 1) nature of the invention; 2) the state of the prior art; 3) working examples; 4) the amount of direction or guidance presented; 5) the breadth of the claims; 6) the relative skill of those in the art; 7) the predictability or unpredictability of the art; and 8) the quantity of experimentation necessary. *Id.*

Appellants request reversal of the enablement rejection, because the Examiner has not properly backed up the assertions of non-enablement with acceptable evidence or reasoning and, further, has failed to give proper weight to the evidence of enablement currently of record. The Examiner focuses primarily on the alleged breadth of claim 67, while giving nearly no weight to the remaining factors.[1] While the burden is squarely on the Examiner to explain why the claims are not enabled, Appellants analyze all of the *Wands* factors below and address the Examiner's assertions regarding breadth therein.

(1) Nature of the invention

The claim recites a method of lowering free fatty acids in an individual by administering a single new chemical entity, 3-(1H-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole—also known as MK-0354—or a pharmaceutically acceptable salt, solvate or hydrate thereof. *See* Semple, *supra*, at 5107 (referring to claimed compound as MK-0354). The method of lowering free fatty acids is a method of treating a specific type of dyslipidemia—that of high levels of free fatty acids (see specification, page 10, lines 14-17). Measurement of free fatty acid levels after administration of MK-0354 is a simple blood test measurement. *See* Semple, *supra*, at 5107 (describing "In vivo Mouse Lipolysis" at column 2); Eseng Lai, et al., *Effects of a Niacin Receptor Partial Agonist, MK-0354, on Plasma Free Fatty Acids, Lipids, and Cutaneous Flushing in Humans*, 2 J. CLINICAL LIPIDOLOGY 375, 376 (2008) (describing Phase I studies). Moreover, utilizing this testing, the claimed compound has been shown to be effective in lowering free fatty acids in mice based on *in vivo* testing (see results for compound **5a** (MK-0354) and in humans based on Phase I clinical trials. *See* Semple, *supra*, at 5103; Lai, *supra*, 375, 381. Hence, the invention works in humans as asserted in the specification. Appellants are at a loss to understand how an enablement rejection can be maintained after recognizing this fact.

[1] During an interview, the Examiner stated that the claim is not enabled, in part, because the method is on a "list" of terms normally deemed by the USPTO to be non-enabled (see Interview Summary of August 31, 2010). This is clearly not a basis for non-enablement as the USPTO does not have statutory or substantive rulemaking authority to set forth "lists" of non-enabled subject matter. Therefore, Appellants will constrain their arguments to *Wands* factors.

<u>(2) State of the art; working examples; and the amount of guidance or direction provided</u>

The state of the art, the working examples, and the amount of direction or guidance provided all weigh in favor of enablement, because: (1) nicotinic acid (niacin) was known at the time of filing to have activity in lowering free fatty acids as recited by claim 67; (2) the murine variant of the known GPR109A receptor was shown to mediate the metabolic effects of nicotinic acid; (3) nicotinic acid was known to bind to and agonize the GPR109A receptor; and (4) the claimed compound also binds to and agonizes the GPR109A receptor. As a result, one of skill in the art would recognize that a compound which agonizes the GPR109A receptor, such as the claimed compounds would be expected to have efficacy in lowering triglycerides and free fatty acids as does nicotinic acid.[2]

As stated in the specification, nicotinic acid (niacin) was known prior to filing to have efficacy in lowering free fatty acids. Specifically, nicotinic acid was known to reduce the level of free fatty acids in wild-type mice. *See* Sorin Tunaru, et al., *PUMA-G and HM74 are Receptors for Nicotinic Acid and Mediate its Anti-Lipolytic Effect*, 9 NAT. MED. 352, 353-354 (2003).

Nicotinic acid was also known prior to filing to bind to the GPR109A receptor—also known as the HM74A receptor and referred to in the specification as the RUP25 receptor. *See* GenBank record for GenBank Accession No. NM_177551 for the nucleotide and GenBank Accession No. NP_808219 for the polypeptide referenced at page 50, lines 1-6 of the specification; *see also*, Alan Wise, et al., *Molecular Identification of the High and Low Affinity Receptors for Nicotinic Acid*, 278 J. BIOLOG. CHEM. 9869, 9874 (2003). The murine homologue of GPR109A is known as PUMA-G. Studies conducted before the date of filing were consistent with PUMA-G mediating the main metabolic effects of nicotinic acid, including lowering free fatty acid levels. *See* Tunaru, *supra*, at 353-354. Hence, there is ample pre-filing evidence tying

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[2] While the compound is a partial agonist, studies have shown it binds to the GPR109A receptor in ^{3}H nicotinic acid competitive binding study and that it has an EC_{50} of 2.3 μM with an efficacy of 72% of nicotinic acid in a hGPR109a (GTPγS assay). *See* Semple, *supra*, at 5103.

the GPR109A receptor to the efficacy of nicotinic acid in lowering free fatty acid levels such as in the claimed methods.

Further, nicotinic acid was known to function as an agonist at the GPR109A receptor. *See* Wise, *supra*, at 9872). Hence, one of skill in the art would expect other agonists of the GPR109A receptor would have efficacy in treating the same disorders treatable by nicotinic acid. As asserted in the specification, the claimed compound in claim 67 binds to and partially agonizes the GPR109A (RUP25) receptor and, therefore, would be expected to have efficacy in lowering triglycerides and free fatty acids as does nicotinic acid (see page 57 of the specification disclosing EC_{50} values for compounds of the invention in a ^{3}H-nicotinic acid binding competition assay). Further, during prosecution, Appellants also provided the Examiner with the Semple reference to corroborate that the claimed compound can agonize the GPR109A receptor. *See* Semple, *supra*, at 5103 (disclosing EC_{50} values for compound **5a** in Table 1 and that compound **5a** had an EC_{50} of 2.3 μM with an efficacy of 72% of nicotinic acid in a hGPR109a (GTPγS assay)).

As the claimed compound can also bind to and partially agonizes the GPR109A receptor, it would also be expected to lower free fatty acid levels in a similar manner to nicotinic acid. Moreover, as described above, this expected activity for the claimed compound has been established definitively through *in vivo* mice studies and Phase I clinical trials in humans. *See* Semple, *supra*, at 5103; Lai, *supra*, 381. Hence, the state of the art, the working examples, and the amount of guidance or direction provided all weigh heavily in favor of enablement.

Despite this clear evidence of enablement, the Examiner disputes that there is any "nexus between the [EC_{50}] data and the method of lowering free fatty acids" (Office Action, June 1, 2010, page 3). In particular, the Examiner notes that the "instantly claimed compounds are partial nicotinic acid agonists" (Office Action, June 1, 2010 page 4). Appellants note, however, that the compounds still bind competitively to the GPR109A receptor in nicotinic acid competition studies. Specifically, the claimed compound (compound **5a**) had an EC_{50} of 2.3 μM in a hGPR109a (GTPγS assay), with an efficacy of 72% of nicotinic acid. *See* Semple, *supra*, at 5103, column 1). In binding studies, the claimed compound (compound **5a**) was also found to be a competitive inhibitor of ^{3}H nicotinic acid binding to hGRP109a (**5a** K_i = 505 nM; nicotinic

acid K_i = 50 nM). *Id.* Further, to the extent that the "nexus" remains unclear, Appellants have provided evidence showing that the claimed compound does lower levels of free fatty acids in both mice and humans. *See* Semple, *supra*, at 5103; Lai, *supra*, 381. Hence, any such doubt raised by the Examiner has been amply rebutted.

(3) The breadth of the claims

In the face of compelling evidence of enablement, including Phase I clinical trials, the Examiner insists that the method is still non-enabled, because of the supposed overbreadth of the claimed method. Appellants strongly disagree that the method is overbroad. Instead, the scope of claim 67 is relatively narrow in scope and fully commensurate with enablement provided by the specification and the corroborating evidence of enablement summarized in Lai and Semple. First, the claimed method recites a single new chemical entity, rather a genus of such compounds. Second, the method is directed to treatment of a very specific type of dyslipidemia—high levels of free fatty acids (see specification, page 10, lines 14-17). Third, measurement of free fatty acid levels before or after administration of the claimed compound is a simple blood test measurement. *See* Semple, *supra*, at 5107. Fourth, the claimed method has been definitively shown to work in humans. *See* Lai, *supra*, at 381. Appellants can see no reason why one of skill in the art would doubt that the claimed compound can lower free fatty acids in the face of Phase I clinical trials showing that it does.

The Examiner, however, alleges that the term "lowering free fatty acid" is "a broad term that has been linked to many different disorders" (Office Action, June 1, 2010, page 3). The Examiner notes that high free fatty acids has been associated with other disorders such as insulin resistance, type 2 diabetes, high LDL-cholesterol, low HDL-cholesterol, high triglyerides, etc. (Office Action, June 1, 2010, page 3). Therefore, the Examiner concludes that the term is "considered broad because of the wide range of disorders that can be treated" (Office Action, June 1, 2010, page 3). The Examiner also notes that the claimed compound did not produce changes in levels of other lipids, such as triglycerides, LDL-cholesterol or HDL-cholesterol (Office Action, June 1, 2010, page 4). Instead, the Examiner states that the claimed compounds "has shown promise in the lowering of free fatty acids connected to dylipidemia, but not high-density cholesterol, low lipoprotein cholesterol, or triglycerides" (Office Action, June 1, 2010,

page 4). Hence, the Examiner insists that the method of lowering free fatty acids should be "associated with a particular disorder and patient population be treated" (Office Action, June 1, 2010, pages 4-5). Apparently, the Examiner's rejection is based upon the belief that the compound may prove useful to treat too many physical maladies. This is not a proper ground for rejection under current patent practice.

Appellants deny that the method is overbroad or needs to be associated with "a particular disorder or patient population". The Examiner appears to be suggesting that the method of lowering free fatty acids must further be "associated with dyslipidemia". However, as discussed above, lowering free fatty acids is a very specific (and narrow) form of dyslipidemia, rather than a separate disorder. Further, Appellants see no reason why the claimed method is somehow overbroad because it may or may not result in certain <u>downstream</u> effects. The Examiner's untenable position appears to be that a method of treatment must also recite all possible downstream effects in order to be enabled. For example, obesity is known to be sometimes linked to a variety of different downstream effects including cardiovascular disease and type 2 diabetes. Under the Examiner's reasoning, a person seeking to claim a method of treating obesity would also have to conduct clinical trials for all possible downstream effects of treating obesity and then recite those effects in the claims (e.g., a method of treating obesity for the downstream effect of reducing type 2 diabetes). Appellants can find no case law that would support this novel position espoused by the Examiner.

Appellants also deny that the method is overbroad for failure to identify a patient population. The evidence of record does not indicate that there was a patient population for which the claimed compound failed to lower free fatty acid levels. Instead, the Phase I clinical results show that the claimed compound (MK-0354) lowered free fatty acid levels in a dose-dependent manner as compared to the placebo group in the individuals tested. *See* Lai, *supra*, at 378-379, 381. Appellants, therefore, see little support for the Examiner's position that a specific patient population must be recited in the claim. Instead, one of skill in the art would recognize that a patient would be a person with abnormally high levels of free fatty acids.

For at least, these reasons, Appellants respectfully assert that the scope of the claimed method is not overbroad and is commensurate with the evidence of enablement.

(4) The relative level of skill in the art

The Examiner acknowledges, and Appellants agree, that the skill in the art is high. This factor also weighs in favor of enablement.

(5) The predictability or unpredictability in the art

As discussed above, the record includes human clinical data establishing the efficacy of the claimed method. Hence, the evidence of record indicates that the claimed method does, in fact, work, notwithstanding the predictability or unpredictability of the art.

(6) The quantity of experimentation necessary

Little experimentation, if any, would be necessary to establish how to use the claimed methods. As summarized above, Appellants have provided both *in vitro* and *in vivo* testing in mice and humans. As such, the quantity of experimentation would not be undue in light of the large amount of guidance and direction provided to one of skill in the art.

As summarized above, each of the Wands factors clearly weighs in favor of enablement. Moreover, the Examiner has failed to back up the allegations of non-enablement with acceptable reasoning or evidence as required under *In re Marzocchi*. For at least these reasons, Appellants respectfully assert that use of the claimed methods would not require undue experimentation and request that the rejection of claim 67 under 35 U.S.C. § 112, ¶ 1 be reversed.

II. The solvates and hydrates of claims 65-67, on appeal, are enabled.

This is an enablement rejection under 35 U.S.C. §112 ¶ 1 with respect to the solvates and hydrates of the single new chemical entity of claims 65-67. As summarized above, an analysis under 35 U.S.C. § 112, ¶ 1 centers on the *Wands* factors. In the instant case, the Examiner has overemphasized the absence of working examples, misconstrued the predictability factor, and given nearly no weight to the remaining factors. When all of the *Wands* factors are properly considered, it is clear that the solvates and hydrates are enabled. Further, the Examiner provides no objective evidence to support the allegations regarding non-enablement, citing only the USPTO's own presentation at Customer Partnership meeting. *See* James Wilson, *Enablement*

for Derivatives of Compositions of Matter, BIOTECHNOLOGY, CHEMICAL, & PHARMACEUTICAL CUSTOMER PARTNERSHIP MEETING, UNITED STATES PATENT & TRADEMARK OFFICE, March 12, 2008, at 15. Finally, two prior Board of Patent Appeals and Interferences decisions further support reversal of the rejection.

A. Prior decisions by the Board of Patent Appeals and Interferences support reversal of the enablement rejection.

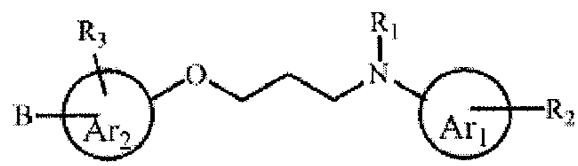
Two recent Board of Patent Appeals and Interferences decisions have held that solvates and hydrates are enabled under factual circumstances similar to the instant case. *See In re Liu*, No. 2009-015302 (Bd. Pat. App. & Int. 9/17/10), *at* http://des.uspto.gov/Foia/ReterivePdf?system=BPAI&flNm=fd2009015302-09-15-2010-1; *In re Germeyer*, No. 2010-005038 (Bd. Pat. App. & Int. 12/3/2010), *at* http://des.uspto.gov/Foia/ReterivePdf?system=BPAI&flNm=fd2010005038-12-01-2010-1. While these decisions are non-precedential, the close factual similarity between the prior and instant cases urges consideration of the decisions as persuasive authority supporting reversal of the instant enablement rejection.

In *Liu*, the Board found the solvates of a genus of compounds enabled, because "the Examiner...overemphasized the importance of working examples, and [gave] too little credit to the abilities of a person having ordinary skill in the art". *Liu*, No. 2009-015302, slip op. at 8. While noting that it might be difficult to predict whether a given compound will form a solvate or hydrate, the Board noted that there was "evidence that solvates and hydrates are routinely produced and characterized routinely". *Id.* Further, the Board dismissed the Examiner's concern that some of the compounds may not have produced solvates, even though they were in contact with solvents. *Id.* Instead, the Board noted that the "conditions...were unfavorable for solvate formation and, therefore, not indicative of the nonexistence of solvates". *Id.* at 9.

Similar to *Liu*, the Examiner in the instant case has also overemphasized the lack of working examples and the predictability of the art, while giving insufficient weight to the other *Wands* factors. As summarized below, Appellants have provided evidence that solvates can be routinely produced and characterized as was shown in *Liu* (see section B(1) below)). Further, similar to *Liu*, there is no evidence that the instant claimed species was synthesized under

conditions favorable for solvate formation. Accordingly, the Examiner's allegations regarding the non-existence of the claimed solvates and hydrates in the instant case is without any corroboration as in *Liu* (see section B(6) below).

In addition, Appellants submit that the instant case presents an even stronger case of enablement than in *Liu*. While not emphasized in the Board's decision, the claims under appeal in *Liu* were directed to a genus of compounds, with a variety of choices for the substituent variables:

(I) 

Id. at 2. Despite the number of species presumably encompassed by the genus, the Board found the claims enabled. By contrast, the instant case is directed to a single new chemical entity and its salts (see section B(2) below). The quantity of experimentation and the breadth of the claims are clearly lower than in *Liu*. Hence, the instant case presents a stronger case of enablement.

In the second Board decision in *Germeyer*, the Board found the claimed hydrates enabled, despite the Examiner's focus on the difficulty of predicting the structure of a hydrate in advance of any screening. *Germeyer*, No. 2010-005038, slip op., at 5-6. In rendering its decision, the Board noted that the claims did not recite a hydrate with a specific structure. *Id.* at 6. Further, the Board found there was evidence that hydrates form naturally, whether or not their structure can be predicted in advance. *Id.* Hence, the Board found the Examiner's emphasis on predicting hydrate structure to be "misdirected". *Id.*

Similar to *Germeyer*, the Examiner in the instant case has overemphasized the importance of predicting a hydrate or solvate structure. In particular, the Examiner alleges that the specification is deficient because it "does not set forth in full, clear, and exact terms the identity and locations of the modifications of the compound" (Office Action, October 19, 2009, page 5) (see section B(5) below). As in *Germeyer*, this concern should have no bearing on the instant claims, as they do not recite a hydrate or solvate with a specific structure. In sum, the factual similarities between *Liu* and *Germeyer* urge reversal of the instant rejection.

B. An analysis of the *Wands* factors indicates that the solvates and hydrates can be made without undue experimentation

(1) State of the prior art; and the amount of direction or guidance presented

The Examiner has given short shrift to these factors and has failed to address the evidence provided by Appellants regarding the routine state of the art methods for making the claimed solvates and solvates. With regard to the state of the art, the Examiner merely acknowledges that "[i]t was known in the art at the time of this application that compounds can exist in salt, solvate and hydrate form" (Office Action, June 24, 2008, page 9). Far from suggesting non-enablement, this statement acknowledges that solvates and hydrates of compounds were known in the state of the art. In fact, the Examiner's sole citation in support of the enablement rejection states that "[i]t has been estimated that approximately one-third of pharmaceutically active substances are capable of forming hydrates". *See* Wilson, *supra*, at 15. The solvate and hydrate section of the USPTO's presentation is glaringly devoid of citation to any reference by one of skill in the art. Therefore, it is difficult to determine the factual accuracy of anything in the presentation. However, even accepting *arguendo* that the USPTO's presentation should be given any weight in the enablement analysis, the Examiner's own citation suggests the ubiquity of solvated forms of compounds in the state of the art and, if anything, weighs in favor of enablement, rather than against it. Further, at least one other reference suggests that hydrates and solvates are not rare and are often formed from pharmaceutical compounds:

> Simply exposing an anhydrous powder to high relative humidity can often lead to formation of a hydrate.
>
> More than 90 hydrates are described in various USP monographs.
>
> Often, when solvents are employed in the purification of new drug substances by recrystallization, it is observed that the isolated crystals include solvent molecules, either entrapped within empty spaces in the lattice or interacting via hydrogen bonding or van der Waals forces with molecules constituting the crystal lattice.

J. Keith Guillory, *Generation of Polymorphs, Hydrates, Solvates, and Amorphous Solids, in* POLYMORPHISM IN PHARMACEUTICAL SOLIDS, 183, 202, 204-05 (Harry G. Britain, ed., 1999).

Secondly, with respect to the amount of direction or guidance presented, the Examiner has failed to consider the evidence provided by Appellants regarding the well known and routine methods for preparing solvates and hydrates. Instead, the Examiner alleges incorrectly that "[t]here is no guidance in the specification drawn to the solvates and hydrates of the instantly claimed compounds", thereby ignoring what was well known in the art at the time of filing (Office Action, June 24, 2008, page 10). As will be appreciated, the specification "need not teach, and preferably omits, what is well known in the art". *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). Appellants note that there were routine methods for making and characterizing the solvates and hydrates of compounds, which were well known at the time of filing. *See e.g.*, Guillory, *supra*, at 183-226. For example, section I of Guillory describes methods employed to obtain unique polymorphic forms, while sections II and III describe more specific methods of preparing hydrate and solvate forms, respectively. *Id.* In particular, section II notes that hydrates can be formed routinely by recrystallization from water or mixed aqueous solvents or, in some instances, by simple exposure to an atmosphere containing water vapor. *Id.* at 203-04. Similarly, section III describes techniques for obtaining solvates, including crystallization from a single solvent or mixed solvent, vapor diffusion, or solvent exchange. *Id.* at 207. Guillory further describes the synthesis of several known pharmaceutical solvates and hydrates using these techniques and their characterization through methods such as x-ray powder diffraction. *Id.* at 202-08. Guillory notes in conclusion that:

> The pharmaceutical development scientist who is assigned the task of demonstrating that a substance exhibits only one crystalline form, or that of discovering whether additional forms exist, can utilize the techniques outlined in this chapter as a starting point. Upon completion of this program, one can certainly conclude that due diligence has been employed to isolate and characterize the various solid-state forms of any new chemical entity.

Id. at 219. Hence, the evidence of record clearly indicates that, at the time of filing, there were routine methods for preparing hydrates and solvates, as well as empirical methods for

determining whether a pharmaceutical compound may form a solvate or hydrate.[3] The Examiner has not provided any evidence demonstrating that these methods are not routine and applicable to any pharmaceutical compounds, such as the species of claims 65-67. Accordingly, the state of the art and the guidance provided thereby weigh in favor of enablement.

(2) The breadth of the claims

The claims under appeal are relatively narrow in scope, such that one of skill in the art could practice the claimed invention without undue experimentation. Claims 65-67 recite a single new chemical entity, 3-(1H-tetrazol-5-yl)-2,4,5,6-tetrahydro-cyclopentapyrazole, and its pharmaceutically acceptable salts, solvates, and hydrates. At issue is the enablement of the recited solvates and hydrates. One of skill in the art, armed with the empirical methods of screening detailed above, could readily engage in routine experimentation to determine what solvates and hydrates are formed by this single new chemical entity and its salts without resorting to undue experimentation. It is well-established that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening". *In re Wands*, 858 F.2d 731, 736-37, 8 U.S.P.Q.2d 1400, 1401 (Fed. Cir. 1988). Instead, the touchstone is undue experimentation. It is difficult for Appellants to conceive of how undue experimentation would be required to screen a single new chemical entity and its salts for the formation of the claimed solvates and hydrates, considering the routine methods of screening known in the art and the high skill of a person of skill in the art.

(3) The relative skill of those in the art

The Examiner acknowledges that the skill in the art is high (Office Action, June 24, 2008). Appellants note that a person of skill in the art would be aware of the routine methods of preparing and characterizing solvates and hydrates as summarized above. Hence, this factor also weighs in favor of enablement.

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[3] In their response to the Office Action of June 24, 2008, Appellants further noted that there are numerous companies that routinely provide screening for solvates and hydrates, Wilmington PharmaTech (Wilmington, DE) and Avantium Technologies (Amsterdam). This observation remains uncontested by the Examiner.

(4) The quantity of experimentation necessary

One of skill in the art could make the claimed solvates and hydrates without undue experimentation, considering the state of the art, the guidance provided thereby, the breadth of the claims, and the relative level of skill in the art. As will be appreciated, the test for whether experimentation would be undue is not merely quantitative since a considerable amount of experimentation is permissible, if it is merely routine. *Wands*, 858 F.2d at 736-37, 8 U.S.P.Q.2d at 1404. The instant case can be directly contrasted with the assay methods in *Wands*. In *Wands*, the Office had rejected the appealed claims, directed to methods for assaying HBsAg using high-affinity IgM monoclonal antibodies, as lacking enablement. *Wands*, 858 F.2d at 734, 8 U.S.P.Q.2d at 1402. The Office alleged that the production of high-affinity IgM anti-HBsAg antibodies was unpredictable and unreliable and, therefore, would require undue experimentation. *Wands*, 858 F.2d at 735, 8 U.S.P.Q.2d at 1402. The Federal Circuit disagreed, finding that undue experimentation would not be required. *Wands*, 858 F.2d at 739-40, 8 U.S.P.Q.2d at 1406. Even though screening for hybridomas involved several, labor-intensive steps (see the steps in Table 1), the court found that this amount of effort was not excessive or undue, as the methods needed to practice the invention were well-known and the level of skill in the art was high. *Wands*, 858 F.2d at 737-40, 8 U.S.P.Q.2d at 1404-06. The court noted that a finding of undue experimentation would not be required even if the success rate for producing the antibodies was only 2.8% as suggested by the Office (as contrasted with the 44% success rate advanced by the applicant). *Wands*, 858 F.2d at 739-40, 8 U.S.P.Q.2d at 1405-06.

In stark contrast with the antibody-making procedures at issue in *Wands*, the preparation of hydrates and solvates of a particular organic molecule is an easier and simpler process, which requires significantly fewer steps and less time than the preparation of a monoclonal antibody. Table 1 provides a step-by-step comparison of some of the major steps involved in the production of a monoclonal antibody (as disclosed in *Wands*) and the one step involved in making a hydrate or solvate. To make hydrates and solvates, samples of the organic compound are exposed to water or various different solvents. *See e.g.*, Guillory, *supra* at 203-04, 207 (describing methods including recrystallization with a single solvent or mixed solvent; or through vapor diffusion). Once the hydrates and solvates are formed, they can be readily

analyzed by routine methods or other routine techniques such as X-ray powder diffraction. *See* Guillory, *supra*, at 202-08. Further, this routine synthesis would be significantly less arduous given the narrow breadth of the claims, directed to a <u>single</u> new chemical entity and its salts. As is clearly shown in Table 1 and summarized above, the production of a monoclonal antibody is significantly more complex and time-consuming than the production of a hydrate or solvate, yet the *Wands* court concluded that the production of a monoclonal antibody was not excessive and undue. It is clearly inconsistent for the Examiner to allege that the production of hydrates and solvates would require undue experimentation, while the production of monoclonal antibodies would not require undue experimentation. Hence, this factor clearly weighs in favor of enablement.

Table 1

Step	Monoclonal Antibody	Hydrate or Solvate
1	immunize animal	recrystallization from a single or mixed solvent; or vapor diffusion
2	remove the spleen from the immunized animal	
3	separate the lymphocytes from the other spleen cells	
4	mix the lymphocytes with myeloma cells	
5	treat the mixture to cause fusion between the lymphocytes and the myeloma cells to make hybridomas that hopefully secrete the desired antibody	
6	separate the hybridoma cells from the unfused lymphocytes and myeloma cells by culturing in a medium in which only hybridoma cells survive	
7	culture single hybridoma cells (often 100 of different cells) in separate chambers	
8	assay the antibody secreted from each hybridoma culture to determine if it binds to the antigen	

<u>(5) The nature of the invention</u>

Considering the state of the art, the guidance presented therein and the skill of a person of skill in the art, there is nothing in the nature of the invention which would preclude enablement. However, the Examiner makes two unsubstantiated statements regarding the nature of the invention. First, the Examiner alleges that the hydrates and solvates encompass "a class of compounds that have different activity from regular compounds" (Office Action, March 18, 2009, page 4). The Examiner appears to be alleging that hydrates and solvates would somehow not retain the same pharmacological properties of the claimed species. This unsubstantiated allegation runs counter to what is known in the art regarding pharmaceutically acceptable solvates and hydrates. A hydrate or solvate retains the base structure of the species, but incorporates water or solvent into the crystal lattice of the compound in stoichiometric proportions. *See* Guillory, *supra*, at 202, 205. In other words, the water or solvent molecules are not bonded through covalent or ionic bonds to other elements of the molecule. One of skill in the art would recognize that these water or solvent molecules will be lost *in vivo*, once the molecule is dissolved in bodily fluids, as the solid state crystal lattice is no longer present. There is, therefore, little reason to suspect that the solvates and hydrates would somehow not have pharmacological activity of the unsolvated compound, since it will become unsolvated compound *in vivo*. Further, Guillory describes hydrates and solvates of a number of pharmaceutical compounds, which further undercuts the Examiner's allegation. *Id.* at 202-08. Moreover, the Examiner simply has provided no evidence to support this radical stance. Hence, the record does not support this factor weighing in favor of non-enablement.

Second, the Examiner alleges that "[i]t is not the norm that one can predict with any accuracy [that] a particular solvate form of an active compound will be <u>more</u> soluble, <u>more</u> easily handled in formulations or <u>more</u> bioavailable without actual testing in vivo" (Office Action, June 24, 2008, page 8 (emphasis added)). The Examiner seems to be suggesting that the standard for enablement should be higher for the solvated forms of the compound, than for its unsolvated form. Appellants are aware of no case that would stand for this proposition. There simply is no "super enablement" requirement for solvates and hydrates which would mandate that the claimed solvates and hydrates be <u>more</u> bioavailable, <u>more</u> soluble, or <u>more</u> easily handled than the

unsolvated form. Appellants cannot discern why this statement by the Examiner has any relevance whatsoever to the enablement inquiry.

Finally, the Examiner also alleges that the specification is deficient, because it "does not set forth in full, clear, and exact terms the identity and locations of the modifications of the compound" (Office Action, October 19, 2009, page 5). The Examiner further alleges that it is "known that crystalline states of compounds such as solvates and hydrates can undergo phase transformation and that an exact disclosure of the changes should be disclosed" (Office Action, page 5). While it is a bit unclear, the Examiner's reference to the "identity and locations of the modifications of the compound" presumably refers to stoichiometry of the solvates and hydrates and/or the exact crystal structure of the solvates and hydrates. This statement by the Examiner appears to be nothing more than an insistence on working examples, which is addressed below. Moreover, the claims do <u>not</u> require a particular solvate or hydrate structure (e.g., a monohydrate, dihydrate, hemihydrate, etc.), but, rather, recite hydrates and solvates more generally. It is well-established that an applicant need only enable the <u>claimed</u> subject matter.

<u>(6) The presence or absence of working examples</u>

The Examiner improperly insists that the solvates and hydrates can only be enabled if working examples have been described by the specification, effectively ignoring the routine nature of solvate and hydrate formation and the relative skill in the art. It is established that there is no requirement for working examples "if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without an undue amount of experimentation". *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (C.C.P.A. 1970). As described above, the state of the art, the guidance provided thereby, the breadth of the claims, the relative level of skill in the art, and the quantity of experimentation all clearly point to routine experimentation, rather than undue experimentation being needed. Under these circumstances, the Examiner has put undue weight on the absence of working examples.

Further, the Examiner leaps to the erroneous conclusion that the claimed solvates and hydrates cannot be formed under any circumstances, alleging that the "examples presented all fail to produce a solvate or hydrate" (Office Action, June 24, 2009, page 8). The specification, however, does not contain any description of using the routine methods in Guillory, such as

recrystallization or vapor diffusion, to prepare a solvate or hydrate of the claimed species or its salt. Instead, the specification only describes the synthesis of the species itself under conditions unfavorable to the formation of a solvate or hydrate—i.e., with removal of solvent under reduced pressure and/or the concentration of the solution to yield the claimed compound (specification, at page 59, line 13 through page 65, line 22). The solvent removal conditions are not favorable to the formation of hydrates and solvates as are the methods described in Guillory. Hence, the Examiner has not pointed to any evidence that the Applicants prepared the base species under conditions that would be favorable for forming hydrates or solvates (such as the use of the methods in Guillory), such that a conclusion could be drawn about the ability of the species to form solvates or hydrates. Further, Guillory points out that investigation of solid state forms such as hydrates and solvates is important during clinical development to obtain regulatory approval of a drug candidate. *See* Guillory, *supra*, at 184-186. The examples of the instant application, in contrast, were performed earlier in the drug development process—during drug discovery—when the focus is on optimizing the pharmacological activity of the compounds and the formation of different solid state forms is not a concern. The methods usually used for purification of compounds in drug discovery, as described in the examples (typically by chromatography followed by evaporation of the product-containing fractions under reduced pressure) do not involve crystallization or vapor diffusion under the conditions favorable to forming hydrates and solvates such as in Guillory. Hence, the Examiner's speculative conclusion that the claimed solvates and hydrates do not exist is lacking any merit.

The Examiner also incorrectly likens the instant case to that in *Morton International Inc. v. Cardinal Chemical Co.*, 5 F.3d 1464, 28 U.S.P.Q.2d 1190 (Fed. Cir. 1993). However, the facts of *Morton* are clearly not analogous to the facts of the instant case. In *Morton*, there was clear and convincing evidence that synthetic methods described in the examples did not produce the claimed compounds. *Morton*, 5 F.3d at 1469-70, 28 U.S.P.Q.2d at 1194. By contrast, in the instant case, the Examiner has provided absolutely no evidence—much less, clear and convincing evidence—that the routine methods described in Guillory would not provide the claimed solvates and hydrates. Indeed, as summarized above, no part of the specification describes the failure of screening methods to provide a solvate or hydrate of the base species. The Examiner's analogy to *Morton*, therefore, is severely lacking.

(7) The predictability or unpredictability of the art

The Examiner overemphasizes the unpredictability of the art, without giving sufficient weight to the remaining *Wands* factors. The Examiner alleges—without citation to any scientific reference—that the "predictability of the arts with regard to salts is known, but the preparation of solvates and hydrates is compound specific" and, therefore alleges that "nothing short of extensive testing...would be needed to determine if additional derivatives exist" (Office Action, June 24, 2008, page 10). Even assuming that one cannot predict beforehand, without conducting any experimentation, whether a given compound will form a hydrate or solvate, this alone would not preclude enablement. It is established that the existence of routine methods of screening can enable a claimed invention even in an unpredictable art. *See e.g., In re Angstadt*, 537 F.2d 498, 502-03, 190 U.S.P.Q. 214, (C.C.P.A. 1976) (finding an unpredictable catalytic process was enabled because each potential catalyst could readily be tested in the process); *Wands*, 858 F.2d at 739-40, 8 U.S.P.Q.2d at 1406 (finding production of high-affinity IgM anti-HBsAg antibodies having only a success rate of 44% was enabled, due in part to the existence of routine methods of synthesis). As noted above, routine, empirical methods exist for determining whether a particular pharmaceutical compound can form a solvate or hydrate. Guillory indicates that use of the routine, empirical methods is a reliable way of screening for solvate and hydrate forms. *See* Guillory, *supra*, at 219 (stating that "[u]pon completion of this program, one can certainly conclude that due diligence has been employed to isolate and characterize the various solid-state forms of any new chemical entity"). Further, as described above, the amount of experimentation for the instant case would be very low due to the relative skill in the art and the narrow breadth of claims—directed to a single new chemical entity and its salts. Appellants submit that undue experimentation would simply not be required for the instant case.

Further, as summarized above, the state of the art indicates that solvates and hydrates are, in fact, quite commonly formed. Moreover, the Examiner has produced no evidence which would suggest that the claimed species is less likely to produce solvates and hydrates than other pharmaceutical substances. Hence, even if one cannot predict beforehand whether a given compound will form a solvate or hydrate, there is nothing in the record which would suggest that one of skill in the art could not simply utilize the routine methods of experimentation to screen the single new chemical entity and its salts for solvate and hydrate formation.

The Examiner's overemphasis on the predictability of the art would essentially make <u>any</u> experimentation undue. The Examiner appears to be insisting that one of skill in the art be able to ascertain, before performing any screening, whether a compound forms a solvate or hydrate. This reasoning was firmly rejected by the court in *Angstadt*:

> If Rainer stands for the proposition that the disclosure must provide "guidance which will enable one skilled in the art to determine, with reasonable certainty before performing the reaction, whether the claimed product will be obtained"... as the dissent claims, then all "experimentation" is "undue," since the term "experimentation" implies that the success of the particular activity is uncertain.

Angstadt, 537 F.2d at 503, 190 U.S.P.Q. at 218-219. Further, this would inevitably force an applicant to screen every species prior to filing and claim only the particular solvate and hydrate forms discovered after such screening. In other words, an applicant seeking to patent a solvate or hydrate would have to provide a screening of each species within a genus of compounds. This is clearly not the law. *Id.* at 502-03 (finding that enablement does not require a disclosure of each species even in an unpredictable art).

In conclusion, because the state of art, the guidance provided thereby, the relative skill in the art, the nature of the invention, the breadth of the claims, and the quantity of experimentation needed weigh in favor of enablement, Appellants respectfully request reversal of the rejection of claims 65-67 under 35 U.S.C. § 112, ¶ 1.

Please apply the brief fee of $540 and any other charges or credits to Deposit Account No. 06-1050, referencing attorney docket number 22578-0005US1.

Respectfully submitted,

Date: March 30, 2011

Susanne H. Goodson, Ph.D., J.D.
Reg. No. 58,450

Customer Number 26204
Fish & Richardson P.C.
Telephone: (302) 652-5070
Facsimile: (877) 769-7945

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Appendix of Claims

65. A compound, which is 3-(1H-tetrazol-5-yl)-2,4,5,6-tetrahydro-cyclopentapyrazole, or a pharmaceutically acceptable salt, solvate or hydrate thereof.

66. A pharmaceutical composition comprising 3-(1H-tetrazol-5-yl)-2,4,5,6-tetrahydro-cyclopentapyrazole, or a pharmaceutically acceptable salt, solvate or hydrate thereof, in combination with a pharmaceutically acceptable carrier.

67. A method of lowering free fatty acids in an individual comprising administering to said individual a therapeutically-effective amount of 3-(1H-tetrazol-5-yl)-2,4,5,6-tetrahydro-cyclopentapyrazole, or a pharmaceutically acceptable salt, solvate or hydrate thereof.

Related Proceedings Appendix

NONE.

Evidence Appendix

Each item of evidence is detailed below, along with a description of when the evidence entered the record. Copies of each item directly follow.

1. Graeme Semple, et al., *3-(1H-Tetrazol-5-yl)-1,4,5,6-Cyclopentapyrazole (MK-0354): A Partial Agonist of the Nicotinic Acid Receptor, G-Protein Coupled Receptor 109a, with Antilipolytic But No Vasodilatory Activity in Mice*, 51 J. MED. CHEM. 5101, 5101-5108 (2008).
   -cited by Appellants on August 18, 2009; considered by Examiner on October 19, 2009

2. Eseng Lai, et al., *Effects of a Niacin Receptor Partial Agonist, MK-0354, on Plasma Free Fatty Acids, Lipids, and Cutaneous Flushing in Humans*, 2 J. CLINICAL LIPIDOLOGY 375, 375-383 (2008).
   -cited by Appellants on April 16, 2010; considered by Examiner in Office Action of June 1, 2010

3. Sorin Tunaru, et al., *PUMA-G and HM74 are Receptors for Nicotinic Acid and Mediate its Anti-Lipolytic Effect*, 9 NAT. MED. 352, 352-355 (2003).
   -cited by Appellants on December 17, 2008; considered by Examiner on March 18, 2009

4. GenBank Accession No. NM_177551
   -provided with response filed on December 17, 2008 (in PAIR: Applicants Arguments/Remarks made in an amendment)

5. GenBank Accession No. NP_808219
   -provided with response filed on December 17, 2008 (in PAIR: Applicants Arguments/Remarks Made in an Amendment)

6. Alan Wise, et al., *Molecular Identification of the High and Low Affinity Receptors for Nicotinic Acid*, 278 J. BIOLOG. CHEM. 9869, 9869-9874 (2003).
   -cited by Appellant on April 5, 2006; considered by Examiner on January 1, 2008

7. James Wilson, *Enablement for Derivatives of Compositions of Matter*, BIOTECHNOLOGY, CHEMICAL, & PHARMACEUTICAL CUSTOMER PARTNERSHIP MEETING, UNITED STATES PATENT & TRADEMARK OFFICE, March 12, 2008, at 1-27.
   -cited by Examiner on October 19, 2009

8. J. Keith Guillory, *Generation of Polymorphs, Hydrates, Solvates, and Amorphous Solids, in* POLYMORPHISM IN PHARMACEUTICAL SOLIDS, 183-226 (Harry G. Britain, ed., 1999).
   -cited by Appellant on December 17, 2008; considered by Examiner on March 18, 2009

Journal of
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Article

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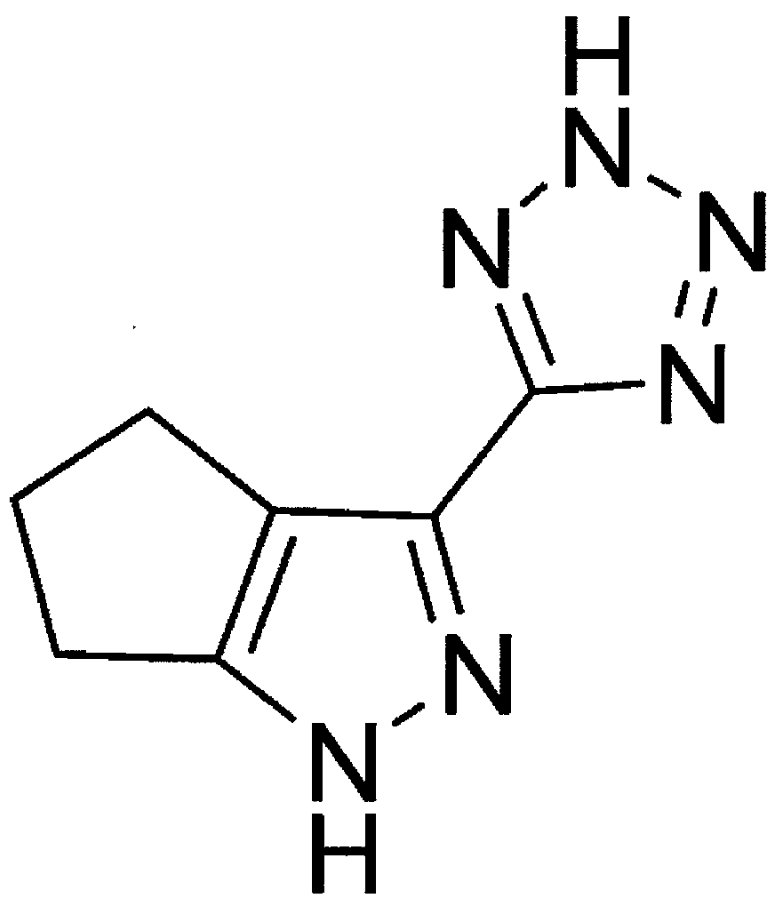

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Graeme Semple,[*,†] Philip J. Skinner,[†] Tawfik Gharbaoui,[†] Young-Jun Shin,[†] Jae-Kyu Jung,[†] Martin C. Cherrier,[†] Peter J. Webb,[†] Susan Y. Tamura,[†] P. Douglas Boatman,[†] Carleton R. Sage,[†] Thomas O. Schrader,[†] Ruoping Chen,[‡] Steven L. Colletti,[§] James R. Tata,[§] M. Gerard Waters,[§] Kang Cheng,[§] Andrew K. Taggart,[§] Tian-Quan Cai,[§] Ester Carballo-Jane,[§] Dominic P. Behan,[‡] Daniel T. Connolly,[‡] and Jeremy G. Richman[‡]

Departments of Medicinal Chemistry and Discovery Biology, Arena Pharmaceuticals, 6166 Nancy Ridge Drive, San Diego, California 92121, Merck Research Laboratories, Rahway, New Jersey 07065

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The discovery and profiling of 3-(1*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (**5a**, MK-0354), a partial agonist of GPR109a, is described. Compound **5a** retained the plasma free fatty acid lowering effects in mice associated with GPR109a agonism, but did not induce vasodilation at the maximum feasible dose. Moreover, preadministration of **5a** blocked the flushing effect induced by nicotinic acid but not that induced by PGD_2. This profile made **5a** a suitable candidate for further study for the treatment of dyslipidemia.

Introduction

Nicotinic acid (sometimes called niacin) is a water-soluble vitamin that at high doses in humans favorably modulates essentially all serum lipid and lipoprotein parameters. As a result, nicotinic acid has been used for the treatment of cardiovascular disease for many years.[1] Nicotinic acid can lower very low-density lipoprotein cholesterol (VLDL-c),[a] low-density lipoprotein cholesterol (LDL-c), and lipoprotein(a) (Lp(a)), but the recent upsurge in interest in this area has focused on nicotinic acid's ability to increase high density lipoprotein-cholesterol (HDL-c) to a greater extent than other currently marketed drugs, as HDL-c levels are inversely correlated with the risk of coronary heart disease.[2] Indeed, in the Coronary Drug Project, nicotinic acid was shown to reduce the number of cardiac events over a six-year dosing period and to reduce all cause mortality by 11% after 15 years.[3,4] Subsequently, combinations of nicotinic acid with the LDL-lowering statin class of drugs have been shown to slow the progression of atherosclerosis, decrease the number of cardiac events, and provide a therapeutic benefit beyond that of statins alone.[5,6]

The use of nicotinic acid as a therapeutic, however, is limited by a number of associated side-effects, most notably a highly uncomfortable cutaneous flushing response that generally manifests itself on the upper body and face which can limit patient compliance.[7] Hence the development of novel agents with nicotinic acid-like effects on plasma lipid parameters and atherosclerosis, but that do not induce flushing, has been considered to be a high value goal for some time.

Mechanistic investigations showed that nicotinic acid binds to a G-protein coupled receptor (GPCR) expressed in rat spleen and adipocytes,[8] a finding that sparked a resurgence in the field. Two G_i-coupled orphan GPCRs that share 95% identity and that are both expressed in human adipocytes were subsequently cloned and identified as putative molecular targets for nicotinic acid.[9] GPR109a (also called HM74a) is the human orthologue of the previously described mouse receptor (PUMA-G, called mGPR109a hereafter),[10] whereas GPR109b (also called HM74) differs from hGPR109a and mGPR109a mainly in the intracellular C-terminal tail portion of the receptor and is not expressed in rodents.[9] Nicotinic acid was shown to activate hGPR109a in a guanine nucleotide exchange assay and displace ^{3}H-nicotinic acid from hGPR109a expressing CHO cell membranes with activity in the tens of nanomolar range, but is a much weaker ligand for GPR109b.[9] Further evidence in mGPR109a knockout mice has demonstrated that the free fatty acid (FFA) and triglyceride lowering effects of nicotinic acid are ablated in the absence of this receptor.[10] These data, coupled with the highly restricted species expression of GPR109b, has brought hGPR109a to the forefront as the more interesting potential drug target of the two. The question still remains however, as to whether either receptor is the molecular target responsible for the lipid remodeling and antiatherogenic properties of nicotinic acid in humans. The demonstration that other known compounds previously shown to raise HDL in humans, acipimox[11] and acifran,[12] are also agonists for hGPR109a[13] is supportive of this idea, but conclusive evidence is still lacking. A hypothesis has been described whereby the initial activation of GPR109a decreases intracellular cAMP levels in adipocytes, leading to reduced protein kinase A (PKA) activity. This in turn results in a decrease in hormone sensitive lipase activity, thereby reducing intracellular triglyceride (TG) hydrolysis and FFA secretion. It has been further postulated that this decrease in FFA levels directly results in decreased production of TG and VLDL in the liver. The use of knockout mice indicates that these antilipolytic effects of nicotinic acid are receptor dependent.[14] What is much less clear is whether the acute effects on plasma FFA and TG can eventually lead to increases in HDL levels. It has been hypothesized, though, that the reduction in the number

* To whom correspondence should be addressed. Phone: +1 858 453 7200. Fax: + 1 858 453 7210. E-mail: gsemple@arenapharm.com.

† Medicinal Chemistry, Arena Pharmaceuticals.

‡ Discovery Biology, Arena Pharmaceuticals.

§ Merck Research Laboratories.

[a] Abbreviations: GPR109a, G-protein coupled receptor 109a (also known as HM74a); GPR109b, G-protein coupled receptor 109b (also known as HM74); PUMA-G, protein upregulated in macrophages by interferon gamma (the mouse orthologue of GPR109a); VLDL-c, very low-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high density lipoprotein-cholesterol; Lp(a), lipoprotein(a); CHO cells, Chinese hamster ovary cells; TG, triglycerides; FFA, Free fatty acids; cAMP, 3′,5′-cyclic adenosine monophosphate; MAPK, Mitogen activated protein kinase; ip, intraperitoneal; PGD_2, prostaglandin D_2.

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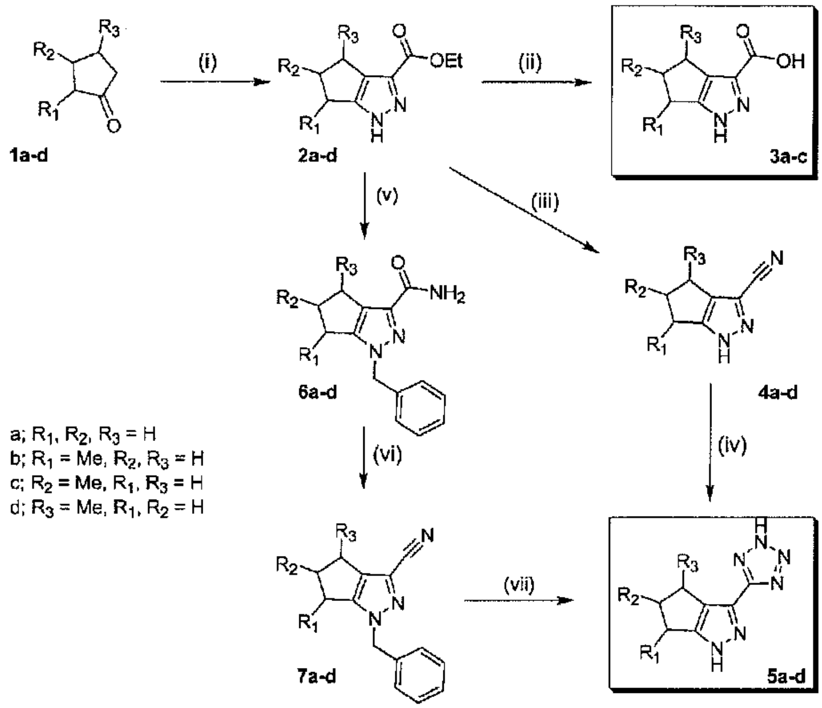
Scheme 1[a]



[a] Reagents and conditions: (i) (a) NaOEt, EtOH, $(EtOCO)_2$, 75 °C; (b) H_2NNH_2 (aq.), 75 °C. (ii) Aqueous LiOH, THF/MeOH, 50−80 °C, 3 h. (iii) (a) NH_3, MeOH, reflux; (b) NaCl, $POCl_3$, MeCN, r.t. 3 h. (iv) NaN_3, DMF, 200 °C, microwave. (v) (a) NH_3, MeOH, reflux *or* NH_4OH, 1,4-dioxane, r.t.; (b) BnBr, base, 1,4-dioxane, r.t. (vi) $SOCl_2$, DMF, r.t. (vii) (a) NaN_3, $ZnBr_2$, DMF 120 °C *or* 200 °C, microwave; (b) DMSO, air, KO^{t}Bu, r.t. *or* HCO_2H/MeOH, Pd (black), r.t.

of VLDL particles may limit the cholesterol ester transfer protein (CETP)-mediated exchange of cholesterol from HDL to VLDL, and TG from VLDL to HDL, thereby leading to a net increase in HDL levels.[14] Clearly the identification of new agonists of the receptor with plasma FFA lowering activity in vivo would be of great interest to further explore this hypothesis. In addition, the identification of compounds that lack the characteristic flushing effect of nicotinic acid may lead to an improvement in patient compliance.

At the outset of our program, it was unclear as to the mechanism of the flushing effect and it appeared possible that the two known pharmacological effects of niacin could be separated with a molecule that selectively activated GPR109a but did not interact with whichever protein was responsible for the flushing. However, it has since been shown that both reduction of plasma FFA and vasodilation by nicotinic acid in mice requires the presence of mGPR109a.[16] From these data, it would appear unlikely that a separation of these two key pharmacological effects can be achieved. Despite this supposition, we herein report the discovery of a new partial agonist of GPR109a that retains the plasma FFA lowering effects in rodents associated with receptor activation but that does not induce any vasodilation in mice at the maximum feasible dose and that acts as a competitive antagonist of nicotinic acid-induced vasodilation in the same model.

Our lead identification strategy, which involved an extensive SAR investigation starting from known small molecule compounds that had been shown to activate the receptor, has been described previously.[17] This in vitro exploration of 5- and 6-membered heterocyclic acids led us to focus on a series of pyrazole acid derivatives. Further screening and elaboration provided a series of 5,5-fused pyrazole acids as suitable lead compounds. We have also demonstrated for a series of 4,5- and 5- substituted pyrazoles, replacement of the acid functionality with a tetrazole resulted in a loss in potency of 1−2 orders of magnitude for GPR109a, although, on the whole, receptor selectivity was maintained.[18] Despite this unpromising precedent, we sought to apply this isosteric replacement to the 5,5-fused pyrazole analogues, and so a series of acids and tetrazoles was prepared as outlined in Scheme 1.

Starting from the appropriate cyclic ketone (**1a−d**), acylation with diethyl oxalate followed by cyclization of the resultant diketo ester with hydrazine provided the bicyclic pyrazole esters **2a−d**. Base catalyzed hydrolysis of the ester function provided the acids **3a−c**. Our original route to the tetrazole series consisted of direct amidolysis of **2a−d** to provide the primary amides that were dehydrated by treatment with $POCl_3$ to provide the nitriles **4a−d** (R = H). Dipolar cycloaddition of **4a−d** with sodium azide under microwave heating provided the tetrazole analogues **5a−d**. All of the compounds containing alkyl groups on the cyclopentyl ring were prepared only as racemates. As a result of the very low yields observed in the latter two steps of the tetrazole synthesis, we also carried out a similar reaction sequence via the benzyl protected pyrazole amides **6a−d**. Dehydration to provide the nitriles **7a−d**, followed by dipolar cycloaddition with sodium azide and benzyl group deprotection, again provided the tetrazole analogues **5a−d**. The addition of these two steps significantly improved the overall yield, but the synthetic sequence was somewhat more cumbersome. For the preparation of **5a** on a larger scale, an alternative route was developed whereby ketone **1a** was acylated with the sodium salt of 1H-tetrazole-5-carboxylic acid ethyl ester and the resultant tetrazole diketone treated with hydrazine to form the pyrazole tetrazole directly.[19]

A comparison of the in vitro agonist activity data of the compounds synthesized is shown in Table 1. The 5,5-bicyclic acid (**3a**)[17] showed good potency and full efficacy at both the cloned hGPR109a and mGPR109a receptors, whereas simple

Table 1. Agonist activity of bicyclic pyrazoles in the whole cell cAMP assay at the homologous hGPR109a and mGPR109a receptors

compound	R_1	R_2	R_3	hGPR109a EC_{50}, μM (n)[a]	efficacy (% nicotinic acid response)	mGPR109a EC_{50}, μM (n)[a]	efficacy (% nicotinic acid response)
3a	H	H	H	0.86 ± 0.05 (2)	106 ± 13	0.5 ± 0.14 (2)	97 ± 4
3b	Me	H	H	8.3 ± 0.2 (3)	93 ± 14	12.2 ± 0.36 (2)	94 ± 5
3c	H	Me	H	n.e.[d]	n.e.	n.e.	n.e.
5a	H	H	H	1.65 ± 0.22 (23) [b]	59 ± 15	1.08 ± 0.33 (16) [c]	71 ± 15
5b	Me	H	H	n.e.	n.e.	n.e.	n.e.
5c	H	Me	H	n.e.	n.e.	n.e.	n.e.
5d	H	H	Me	n.e.	n.e.	n.e.	n.e.

[a] EC_{50} from multiple determinations. Errors shown are ± SEM. [b] 95% confidence interval = 1.3–2.0 μM. [c] 95% confidence interval = 0.7–1.6 μM. [d] n.e. = no effect at maximum concentration tested (100 μM).

methyl substitution around the cyclopentyl ring (**3b**–**c**) gave a significant reduction in potency or ablation of activity. In contrast to our previous experience, when the carboxylic acid moiety was replaced by a tetrazole, the unsubstituted analogue **5a** retained comparable potency to the parent. None of the methyl substituted bicyclic pyrazole tetrazole analogues, however, showed significant receptor activity in the cAMP assay. Furthermore, **5a** demonstrated clear and statistically significant partial agonism in the cAMP assays for both the mouse and human receptors with efficacy approximately 60–70% of that of either nicotinic acid or β-hydroxy butyrate, a putative physiologically relevant ligand for hGPR109a,[20] in the same assay platform. In addition, the compound showed no activation of GPR109b in the cAMP assay at any concentration up to 100 μM. Following these interesting observations, we then prepared a number of other 5,5-fused pyrazoles analogous to those that showed receptor activity in our earlier studies. For example, insertion of either an oxygen or sulfur heteroatom into the 5-membered ring fused to the pyrazole, while maintaining modest activity when the acid functionality was a carboxylate,[17] showed barely measurable activity in the GPR109a cyclase assay when this group was replaced by a tetrazole. Hence, compound **5a** appeared to be somewhat unique among the members of the pyrazole tetrazole series in having reasonable receptor activity. As it was the first compound that we had discovered that showed clear partial agonist character in our in vitro cAMP assays for both hGPR109a and mGPR109a, we selected this compound for more extensive profiling and closer comparison with **3a**.

Further characterization of **5a** in a hGPR109a GTPγS assay (EC_{50} = 2.3 ± 0.4 μM, n = 4; efficacy 72% of nicotinic acid response) confirmed the partial agonist character observed in the cAMP assay, whereas **3a** was again a full agonist in this assay (EC_{50} = 10.4 ± 3.4 μM; n = 4; efficacy 99% of nicotinic acid response). Importantly for future in vivo studies, this partial agonist activity was maintained on the rodent receptors (**5a**; mGPR109a GTPγS assay, EC_{50} = 0.4 ± 0.06 μM; n = 3; efficacy 68% of nicotinic acid response; rat GPR109a GTPγS assay, EC_{50} = 2.3 ± 0.6 μM, n = 3; efficacy 38% of nicotinic acid response). In binding studies, **5a** was a competitive inhibitor of ^{3}H-nicotinic acid binding to hGPR109a (**5a**; K_i = 505 ± 40 nM, n = 6; nicotinic acid; K_i = 50 ± 4 nM, n = 6). In a further demonstration of competition between **5a** and nicotinic acid, **5a** was shown to antagonize the effect of nicotinic acid in the hGPR109a cAMP assay, with a maximum antagonist efficacy consistent with its partial agonist character (see Supporting Information). Despite being a partial agonist of the cAMP pathway, **5a** was able to fully inhibit isoproterenol stimulated lipolysis in human adipocytes (**5a**; IC_{50} = 3.1 ± 0.1 μM, n = 5) as, less surprisingly, was **3a**, and so both of these compounds were progressed into further studies. In line with the hypotheses outlined above, we focused our in vivo screening first on testing

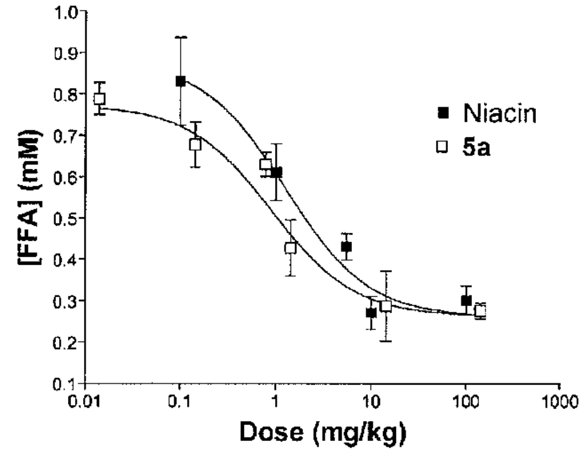


Figure 1. Effect of acute **5a** and nicotinic acid on plasma FFA levels in fasted C57/BL6 mice. Compounds were administered ip in saline 20 min prior to sample collection.

for acute effects on plasma FFA as a potential marker for longer term lipid profile modification and second on characterizing the flushing side effect in a quantitative manner.

The time course of the nicotinic acid-induced plasma FFA reduction was determined in mouse, and dose–response experiments with **5a** were performed at a single time point (20 min), the time of maximum efficacy of nicotinic acid in this model (Figure 1). Compound **5a** was similar in efficacy and marginally more potent than nicotinic acid in this acute test (ED_{50} for **5a** = 3.1 ± 0.9 mg/kg; ED_{50} for nicotinic acid = 9.7 ± 3.6 mg/kg), confirming what we had observed in vitro, that a partial agonist was capable of fully suppressing lipolysis in these systems. It has been previously shown that the antilipolytic effect of nicotinic acid requires the presence of the receptor as the compound has no effect on FFA in mGPR109a knockout mice. Compound **5a** was also without effect on FFA in mGPR109a knockout mice (see Supporting Information). The measured pharmacokinetic parameters in mice (Table 2) were consistent with the observed in vivo pharmacodynamic effect and plasma concentrations taken from the same samples from which plasma FFA measurements were made and further confirmed the pharmacokinetic-pharmacodynamic relationship.

Partial agonists of GPR109a have been previously described along with a prediction that such compounds may have tissue specific effects, but no in vivo data was included.[21] More recently, it has been reported that other agonists of the receptor, both full and partial, including 5-isopropyl pyrazole-3-carboxylate, did not induce flushing in mice.[22] In this paper, it was concluded that some GPR109a agonists may differentially activate parallel downstream receptor signaling pathways and that differences in flushing in vivo were not necessarily a function of efficacy at the receptor. Compounds that inhibit the

Table 2. Pharmacokinetic Parameters in Mouse for **5a**

parameter[a]	
Cl_p (mL/min/kg)	52
V_d (L/kg)	13
$T_{1/2}$ (h)	10
C_{max} (μM)	16
T_{max} (h)	0.083
F_{oral} (%)	93

[a] Cl_p, plasma clearance (blood clearance for mice); V_d, volume of distribution; $T_{1/2}$, terminal half-life; C_{max}, observed maximal plasma concentration following oral dosing; T_{max}, time to reach the C_{max}; F_{oral}, oral bioavailability. IV doses were formulated in PBS and injected at 5.0 mg/kg to male C57BL/6 mice. Peroral doses were formulated in PBS and given by oral gavage at 10 mg/kg.

cAMP pathway were able to inhibit lipolysis. Compounds that stimulated MAPK-induced phosphorylation of ERK1 and ERK2 were able to induce flushing, whereas those compounds that did not signal through MAPK but were still able to inhibit adenylate cyclase did not induce flushing. Consistent with these observations is that the production of PGD_2 requires the generation of arachidonic acid and its subsequent metabolism to prostaglandins, and this process is known to be regulated by activation of the MAPK pathway.[23] In our assays, **3a** but not **5a** was able to activate MAPK signaling in cells, overexpressing either mGPR109a or hGPR109a. In addition, **5a** was also a competitive antagonist of nicotinic acid-induced MAPK signaling in this model, showing that it can occupy the receptor but still fail to initiate signaling through MAPK.[24] Also consistent with these observations is that **5a** did not induce receptor internalization, a process known to be β-arrestin and MAPK-dependent and was able to block nicotinic acid-induced receptor internalization (data not shown). Hence it would be predicted that **3a** but not **5a** would be able to induce flushing in vivo in mice. It is likely that this ability of **5a** to distinguish between receptor activation pathways is due, at least in part, to its partial agonist character.

The vasodilation effect (a component of, and surrogate for, flushing) may be quantified in anesthetized mice by use of a laser-Doppler instrument to measure blood flow changes in the exposed ear.[15] Nicotinic acid induces a dose-dependent increase in blood flow after ip injection in this model (Figure 2a), such that a dose of 100 mg/kg ip results in a 100% increase over baseline blood flow compared to vehicle treatment after 5 min. Compound **3a** also showed a similar dose-dependent effect in mice in the same dose range (data not shown). In contrast, as predicted from the in vitro data, there was no effect of increasing ip doses of **5a**, up to the maximal feasible dose (based on solubility of **5a** in the administration vehicle) of 400 mg/kg (Figure 2). Subsequent analysis of plasma levels of **5a** verified that concentrations of at least 30-fold higher than those that produced a maximal effect in the plasma FFA-lowering model in mouse were achieved (510 $\pm$ 230 μM following a dose of 100 mg/kg ip; plasma levels not measured at 400 mg/kg). In further experiments to characterize the effect of **5a** in this model, the compound was preadministered at 100 mg/kg ip to mice that were challenged 5 min later with a dose of nicotinic acid (30 mg/kg, ip) that normally produces robust vasodilation. A complete inhibition of the expected nicotinic acid-induced flushing effect was observed (Figure 3), consistent with the competitive antagonist effect of **5a** observed on the nicotinic acid-induced activation of MAPK signaling in vitro. As would also be expected, **5a** failed to antagonize flushing induced by PGD_2, which acts downstream of GPR109a.[15]

In summary, we have described the discovery and profiling of 3-(1*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (**5a**, MK-0354). This compound was found to be a partial agonist of GPR109a in all species tested and was shown to compete with nicotinic acid binding and activity in both a conventional radioligand binding assays and in in vitro functional systems measuring cAMP accumulation. In vivo, **5a** possessed plasma FFA lowering effects in mice comparable to those of nicotinic acid. However, in contrast to nicotinic acid and the closely related pyrazole acid analogue **3a**, **5a** did not induce vasodilation in mice even at very high doses and was able to block the vasodilation induced by nicotinic acid in the same model. In addition, **5a** showed no interaction with any other target tested in a panel of over 120 other proteins, including the hERG channel, was not an inhibitor of any of the major CYP isoforms and had no effect in dog cardiovascular or mouse CNS safety pharmacology models (data not shown). From these data, **5a** was identified as a compound of sufficient interest to progress into further pharmacological studies in animals and eventually into human trials, to test the hypothesis that lowering plasma FFA by activation of GPR109a would result in similar HDL-c elevating lowering effect observed with nicotinic acid. Data from these advanced preclinical and clinical studies will be described elsewhere in due course.

Experimental Section

Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a Varian Mercury VX-400 equipped with a four-nucleus auto switchable probe and *z*-gradient or a Bruker Avance-400 equipped with a Quad Nucleus Probe (QNP) or a Broad Band Inverse (BBI) and *z*-gradient. Chemical shifts are given in parts per million (ppm) with the residual solvent signal used as reference. Coupling constants are reported in Hz. NMR abbreviations are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, br = broad. Microwave irradiations were carried out using the Emyrs synthesizer (Personal Chemistry). Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck), and column chromatography was carried out on prepacked silica gel columns using KP-Sil supplied by Biotage. Evaporation was performed in vacuo on a Buchi rotary evaporator. Celite 545 was used for stated filtrations. Strong cation exchange (SCX) columns were purchased from Phenomenex (Strata SCX 55 μm, 70 Å). All other reagents were purchased from Aldrich.

Analytical HPLC/MS was conducted on an AB/MDS Sciex API 150EX mass spectrometer with an electrospray source, using a Shimadzu Inc. LC-10AD VP HPLC-pump, Shimadzu Inc. SCL-10A VP HPLC system controller, Shimadzu Inc. SPD-10A VP UV detector, monitoring at 214 nm, Leap Scientific CTC HTS, PAL autosampler, Analyst 1.2 software and an (a) Alltech Prevail C18 column (5 μ, 50 mm × 4.6 mm), using a gradient of 5% v/v CH_3CN (containing 1% v/v TFA) in H_2O (containing 1% v/v TFA) (t = 0.0 min) gradient to 100% v/v CH_3CN in H_2O (t = 20.0 min), 3.5 mL/min, or (b) Waters YMC ODS-A C18 column (5 μ, 50 mm × 4.6 mm), using a gradient of 5% v/v CH_3CN (containing 1% v/v TFA) in H_2O (containing 1% v/v TFA) (t = 0.0 min) gradient to 95% v/v CH_3CN in H_2O (t = 4.0 min), 3.5 mL/min.

Preparative HPLC was conducted on a Varian Prostar reverse phase HPLC using a Phenomenex Luna C18 column (10 μ, 250 mm × 50 mm), 5% (v/v) CH_3CN (containing 0.1% v/v TFA) in H_2O (containing 0.1% v/v TFA) gradient to 95% CH_3CN, 60 mL/min, λ = 220 nm, or using a Phenomenex Luna C18 column (10 μ, 250 mm × 21.20 mm), 5% (v/v) CH_3CN (containing 0.1% v/v TFA) in H_2O (containing 0.1% v/v TFA) gradient to 95% CH_3CN, 20 mL/min, λ = 220 nm. Compound **3a** was purchased from Fluorochem but for larger amounts was also prepared using the synthesis described below.

General Procedure for the Synthesis of 1*H*-Pyrazole-3-carboxylic acid ethyl esters (2a–d). The appropriate ketone (**1a–d**) was dissolved in ethanol (5 mL/mmol), and diethyl oxalate (1.2 eq.) and

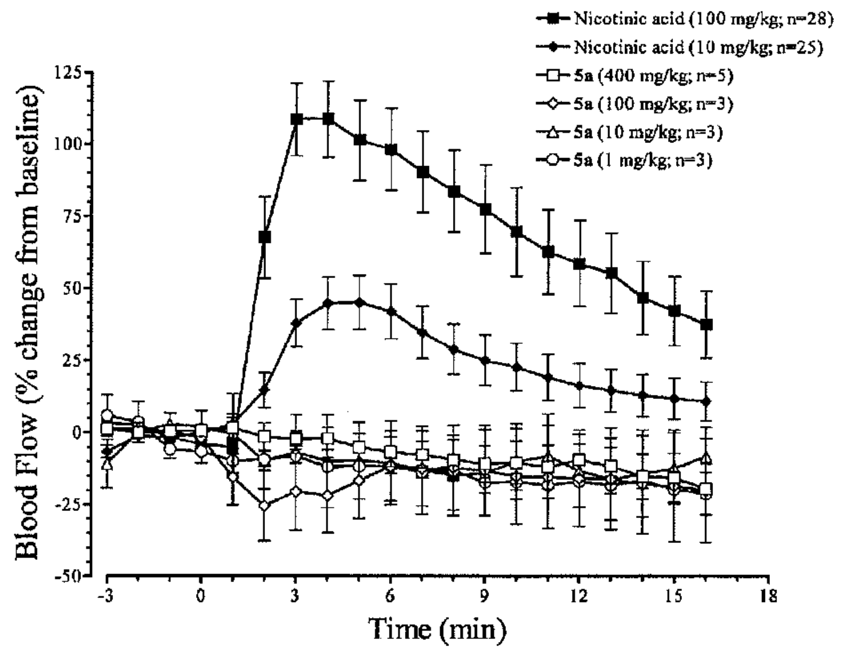


Figure 2. Quantification of the flushing response of nicotinic acid and **5a** as measured by laser-Doppler recordings of blood flow in the ear of male C57/BL6 mice.

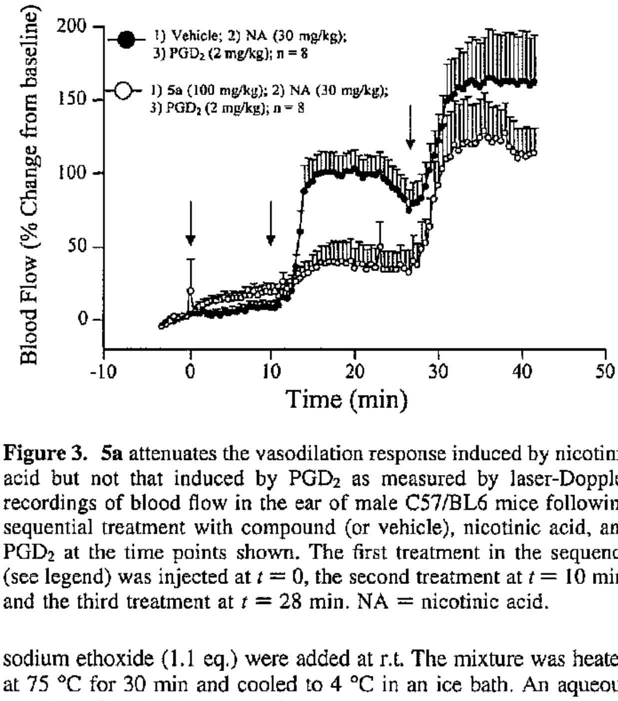


Figure 3. **5a** attenuates the vasodilation response induced by nicotinic acid but not that induced by PGD_2 as measured by laser-Doppler recordings of blood flow in the ear of male C57/BL6 mice following sequential treatment with compound (or vehicle), nicotinic acid, and PGD_2 at the time points shown. The first treatment in the sequence (see legend) was injected at $t = 0$, the second treatment at $t = 10$ min, and the third treatment at $t = 28$ min. NA = nicotinic acid.

sodium ethoxide (1.1 eq.) were added at r.t. The mixture was heated at 75 °C for 30 min and cooled to 4 °C in an ice bath. An aqueous solution of hydrazine (2 equiv, 2 mL/mmol) was added, and the resulting mixture was heated at 75 °C for 1 h. Solvent was removed under reduced pressure, and the crude residue was partitioned between DCM and water. The organic portion was separated and solvent was removed under reduced pressure, and the residue was either purified by column chromatography (*n*-hexane, to 50% EtOAc/*n*-hexane, silica) or hydrolyzed directly to the corresponding 1*H*-pyrazole-3-carboxylic acid (**3**) without further purification or analysis.

1,4,5,6-Tetrahydro-cyclopentapyrazole-3-carboxylic Acid Ethyl Ester (2a). (16.16 g, 90.0 mmol, 76%). *m/z* (ES^+): 181 $[M + H]^+$. ^{1}H NMR (CD_3OD): δ 4.34 (q, 2H, $J = 7.1$, OCH_2CH_3), 2.78 (t, 2H, $J = 7.0$), 2.72 (br s, 2H), 2.49 (br s, 2H), 1.36 (t, 3H, $J = 7.1$, OCH_2CH_3).

6-Methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic Acid Ethyl Ester (2b). (0.603 g, 3.11 mmol, 62%). *m/z* (ES+): 195 $[M + H]^+$, 149 $[M\text{-}OEt]^+$. ^{1}H NMR ($CDCl_3$): δ 10.7 (br s, 1H, N*H*), 4.35 (q, 2H, $J = 7.1$, OCH_2CH_3), 3.16 (sextet, 1H, $J = 7.10$, C(6)-*H*), 2.87–2.77 (m, 1H), 2.77–2.62 (m, 2H), 1.36 (t, 3H, $J = 7.1$, OCH_2CH_3), 1.29 (d, 3H, $J = 6.9$, CH_3). HPLC/MS: (column b) 94%, $t_r = 1.75$ min.

4- and 5-Methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic Acid Ethyl Ester (2c and 2d). (0.570 g, 2.94 mmol, 59%, ca. 1.3:1 mixture of 5-methyl (**2d**) and 4-methyl (**2c**) regioisomers). *m/z* (ES+): 195 $[M + H]^+$, 149 $[M\text{-}OEt]^+$. Major regioisomer 5-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid ethyl ester (**2d**) ^{1}H NMR ($CDCl_3$): δ 10.9 (br s, 1H, N*H*), 4.42–4.30 (m, 2H, OCH_2CH_3), 3.02–2.90 (m, 2H), 2.43–2.32 (m, 2H), 2.08–1.98 (m, 1H), 1.40–1.34 (m, 3H, OCH_2CH_3), 1.21 (d, 3H, $J = 6.3$, CH_3); Minor regioisomer 4-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid ethyl ester (**2c**) *m/z* (ES+): 195 $[M + H]^+$, 149 $[M\text{-}OEt]^+$. ^{1}H NMR ($CDCl_3$): δ 10.9 (br s, 1H, N*H*), 4.42–4.30 (m, 2H, OCH_2CH_3), 3.24 (sextet, 1H, $J = 6.7$, C(4)-*H*), 3.02–2.90 (m, 1H), 2.88–2.60 (m, 3H), 1.40–1.34 (m, 3H, OCH_2CH_3), 1.29 (d, 3H, $J = 6.9$, CH_3).

General Procedure for the Synthesis of 1*H*-Pyrazole-3-carboxylic acids (3a–c). The appropriate pyrazole-3-carboxylate ethyl ester (**2a–c**) was dissolved in a solution of 1:5:1 MeOH:THF:1 M aq LiOH (70 mL) or aq sodium hydroxide and heated to 50–80 °C for 3 h or until hydrolysis was complete. Solvent was removed under reduced pressure, and the resulting solid suspended in water (50 mL). The mixture was acidified to pH 1 by the addition of 1 M HCl and then extracted with ethyl acetate (100 mL). The organic portion was separated and solvent removed under reduced pressure and the residue purified by preparative reverse-phase HPLC to give the pyrazole-3-carboxylic acid (**3a–c**) as an off-white solid.

1,4,5,6-Tetrahydrocyclopenta[*c*]pyrazole-3-carboxylic Acid (3a). (5.10 g, 71%) HPLC/MS: (column a) 99%, $t_r = 2.18$ min, *m/z* (ES+): 153 $[M + H]^+$, 135 $[M\text{-}OH]^+$. ^{1}H NMR (DMSO-d_6): δ 2.70–2.60 (m, 4H, C(4)-*H* and C(6)-*H*), 2.42–2.32 (m, 2H, C(5)-*H*).

6-Methyl-1,4,5,6-tetrahydrocyclopenta[*c*]pyrazole-3-carboxylic Acid (3b). HPLC/MS: (column a) 99%, $t_r = 3.05$ min, *m/z* (ES+): 167 $[M + H]^+$, 149 $[M\text{-}OH]^+$. ^{1}H NMR (DMSO-d_6): δ 2.98 (sextet, 1H, $J = 6.2$, C(6)-*H*), 2.70–2.47 (m, 3H, C(4)-*H* and C(5)-*H*), 1.90–1.80 (m, 1H, C(5)-*H*), 1.11 (d, 3H, $J = 6.9$, CH_3).

5-Methyl-1,4,5,6-tetrahydrocyclopenta[*c*]pyrazole-3-carboxylic acid (3c). HPLC/MS: (column a) 99%, t_r = 2.53 min, *m/z* (ES+): 167 [M + H]$^+$, 149 [M-OH]$^+$. ^{1}H NMR (DMSO-d_6): δ 2.90−2.45 (m, 3H), 2.22−2.12 (m, 2H), 1.07 (t, 3H, *J* = 6.3, C*H*$_3$).

Preparation of 1-Benzyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid amide (6a). 1,4,5,6-Tetrahydro-cyclopentapyrazole-3-carboxylic acid ethyl ester (**2a**) (0.808 g, 4.48 mmol) was suspended in methanolic ammonia (ca. 7 M, 12 mL) and the mixture heated under reflux overnight. The solution was cooled and the resulting precipitate (1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid amide) collected by vacuum filtration as a white crystalline solid (0.438 g, 2.90 mmol, 65%). *m/z* (ES$^+$): 152 [M + H]$^+$. ^{1}H NMR (CD$_3$OD): δ 2.79 (t, 2H, *J* = 6.9), 2.73 (t, 2H, *J* = 7.3), 2.55 (br s, 2H).

To a stirred solution of 1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid amide (3.77 g, 25.0 mmol) in DMF (50 mL) at 25 °C was added K_2CO_3 (12.1 g, 87.4 mmol) followed by benzyl bromide (11.7 g, 62.4 mmol). The reaction was heated to 55 °C and stirred for 16 h. After cooling to ambient temperature, the mixture was diluted with EtOAc (100 mL) and filtered. The filtrate was washed with H_2O (2 × 100 mL), the organic portion dried over $MgSO_4$, filtered, and the solvent removed under reduced pressure. Purification by column chromatography (50−95% EtOAc/hexanes, silica) provided the title compound (1.14 g, 4.73 mmol, 19% yield) as a white solid. *m/z* (ES$^+$): 242 [M + H]$^+$. ^{1}H NMR (CDCl$_3$): δ 7.37−7.30 (m, 3H), 7.19 (m, 2H), 6.67 (br s, 1H), 5.34 (br s, 1H), 5.19 (s, 2H), 2.82 (m, 2H), 2.51 (m, 4H).

Preparation of 1-Benzyl-6-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic Acid Amide (6b). 6-Methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid ethyl ester (**2b**) (0.603 g, 3.11 mmol) was dissolved in 1,4-dioxane (3.5 mL) and ammonium hydroxide (25 mL) added. The resulting solution was stirred overnight at room temperature. Solvent was removed under reduced pressure and the residue dissolved in 1,4-dioxane (30 mL) and 5 M aqueous sodium hydroxide (0.72 mL, 3.64 mmol) added, followed by benzyl bromide (0.56 g, 3.30 mmol). The resulting solution was stirred at 25 °C for 20 h. An additional 5 M aqueous sodium hydroxide solution (0.30 mL, 1.5 mmol) and benzyl bromide (0.25 g, 1.50 mmol) was added, and the solution was stirred at 25 °C for an additional 20 h. Solvent was removed under reduced pressure and the residue partitioned between ethyl acetate and water. The organic portion was separated, solvent removed under reduced pressure, and the resulting residue purified by column chromatography (30−60% EtOAc/hexanes, silica) to provide the title compound (0.470 g, 1.84 mmol, 61% yield) as a colorless oil. HPLC/MS: (column b) t_r = 2.35 min, *m/z* (ES+): 256 [M + H]$^+$, 239 [M-NH$_2$]$^+$. ^{1}H NMR (CDCl$_3$): δ 7.35−7.25 (m, 3H), 7.13 (d, 2H, *J* = 7.2), 6.79 (br s, 1H, CON*H*H), 6.26 (br s, 1H, CONH*H*), 5.21 (q, 2H, *J* = 15.7, C*H*$_2$Ph), 3.00−2.90 (m, 1H), 2.90−2.78 (m, 1H), 2.78−2.65 (m, 2H), 2.10−2.00 (m, 1H), 1.10 (d, 3H, *J* = 6.9, C*H*$_3$). ^{13}C NMR (CDCl$_3$): δ 165.0 (CONH$_2$), 155.4, 138.9, 136.3, 128.7 (C(2′)), 128.5, 127.9 (C(4′)), 126.9 (C(3′)), 54.4 (C*H*$_2$Ph), 40.6 (*C*(5)), 32.0 (*C*(6)), 22.7 (*C*(4)), 19.3 (CH$_3$).

Preparation of 1-Benzyl-5-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic Acid Amide (6c) and 1-Benzyl-4-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic Acid Amide (6d). A mixture of 5- and 4-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid ethyl ester (**2c** and **2d**) (0.570 g, 2.94 mmol) was dissolved in 1,4-dioxane (3.5 mL) and ammonium hydroxide (25 mL) was added. The resulting solution was stirred for 2 days at room temperature. Solvent was then removed under reduced pressure and the residue dissolved in 1,4-dioxane (30 mL) and 5 M aqueous sodium hydroxide (0.72 mL, 3.64 mmol) added, followed by benzyl bromide (0.56 g, 3.30 mmol). The resulting solution was stirred at room temperature for 20 h. Additional 5 M aqueous sodium hydroxide solution (0.30 mL, 1.5 mmol) and benzyl bromide (0.25 g, 1.50 mmol) was added, and the solution was stirred at room temperature for an additional 20 h. Solvent was removed under reduced pressure and the residue partitioned between ethyl acetate and water. The organic portion was separated, solvent removed under reduced pressure, and the resulting residue purified by column chromatography (30−75% EtOAc/hexanes, silica) to give 1-benzyl-4-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid amide **6d** (0.162 g, 0.64 mmol, 21% yield) as a colorless oil. HPLC/MS: (column b) t_r = 2.39 min, *m/z* (ES+): 256 [M + H]$^+$, 239 [M-NH$_2$]$^+$. ^{1}H NMR (CDCl$_3$): δ 7.37−7.26 (m, 3H), 7.17 (d, 2H, *J* = 7.3), 6.73 (br s, 1H, CON*H*H), 5.71 (br s, 1H, CONH*H*), 5.16 (s, 2H, C*H*$_2$Ph), 2.51 (sextet, 1H, *J* = 6.6, C(4)-*H*), 2.75−2.62 (m, 1H), 2.58−2.48 (m, 1H), 2.46−2.35 (m, 1H), 2.10−2.00 (m, 1H), 1.30 (d, 3H, *J* = 6.9, C*H*$_3$). ^{13}C NMR (CDCl$_3$): δ 164.7 (*C*ONH$_2$), 151.2, 139.0, 135.9, 134.1, 128.9 (*C*(2′)), 128.2 (*C*(4′)), 127.7 (*C*(3′)), 55.0 (*C*H$_2$Ph), 39.9 (*C*(5)), 32.4 (*C*(4)), 23.3 (*C*(6)), 21.1 (*C*H$_3$).

Also obtained was 1-benzyl-5-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid amide **6c** (0.213 g, 0.84 mmol, 28% yield) as a white solid. ^{1}H NMR (CDCl$_3$): δ 7.37−7.28 (m, 3H), 7.19−7.14 (m, 2H), 6.68 (br s, 1H, CON*H*H), 5.72 (br s, 1H, CONH*H*), 5.17 (s, 2H, C*H*$_2$Ph), 3.08−2.97 (m, 2H), 2.65 (dd, 1H, J_1 = 15.3, J_2 = 7.5), 2.41 (dd, 1H, J_1 = 18.1, J_2 = 8.7), 2.09 (dd, 1H, J_1 = 15.51, J_2 = 6.2),1.150 (d, 3H, *J* = 6.5, C*H*$_3$). ^{13}C NMR (CDCl$_3$): δ 164.9 (*C*ONH$_2$), 150.9, 139.2, 136.0, 129.0 (*C*(2′)), 128.23 (*C*(4′)), 128.18, 127.7 (*C*(3′)), 55.1 (*C*H$_2$Ph), 41.2 (*C*(5)), 32.7, 32.5, 21.8 (*C*H$_3$).

Preparation of 3-(2*H*-Tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (5a). 1,4,5,6-Tetrahydro-cyclopentapyrazole-3-carboxylic acid amide (0.210 g, 1.39 mmol) was added to anhydrous acetonitrile (12 mL), heated to 80 °C, and sodium chloride (2.0 g, 34 mmol) was added. After 15 min, phosphorus oxychloride (0.128 g, 0.83 mmol) was added and the solution was heated at 80 °C overnight, cooled, filtered, and the collected solid washed with acetonitrile. Solvent was removed from the combined solutions under reduced pressure, and the resulting solid purified by preparative HPLC to provide 1,4,5,6-tetrahydro-cyclopentapyrazole-3-carbonitrile as a deep-purple-colored solid (0.031 g, 0.23 mmol, 17%). *m/z* (ES$^+$): 134 [M + H]$^+$. ^{1}H NMR (CD$_3$OD): δ 2.79 (t, 2H, *J* = 7.3), 2.73 (t, 2H, *J* = 7.1), 2.65−2.55 (m, 2H).

1,4,5,6-Tetrahydro-cyclopentapyrazole-3-carbonitrile (0.022 g, 0.165 mmol) and sodium azide (0.086 g, 1.30 mmol) were dissolved in DMF (3 mL) and heated under microwave irradiation at 175 °C for 20 min. The mixture was cooled to room temperature, filtered, and the filtered solid washed with ethyl acetate. The combined solutions was added to saturated aqueous sodium bicarbonate (20 mL) and washed with ethyl acetate (20 mL). The aqueous layer was acidified to pH 1 with the addition of 1 M aqueous hydrochloric acid and extracted with ethyl acetate (2 × 20 mL). The ethyl acetate extracts were combined and solvent removed under reduced pressure and the resulting solid purified by preparative HPLC to give 3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (**5a**) as a white solid (0.012 g, 0.068 mmol, 41%).

Alternative Preparation of 3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (5a). Thionyl chloride (760 mg, 6.39 mmol) was added dropwise to a solution of 1-benzyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid amide (**6a**, 1.03 g, 4.27 mmol) in DMF (17 mL) at room temperature. The reaction mixture was stirred for 17 h at which time NaHCO$_3$ (saturated aq, 2 mL) was added to quench excess thionyl chloride. The mixture was diluted with EtOAc (100 mL) and washed sequentially with NaHCO$_3$ (saturated aq, 75 mL) and H_2O (2 × 100 mL). The organic portion was separated and dried over $MgSO_4$. The mixture was filtered and solvent removed under reduced pressure to provide 1-benzyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carbonitrile (**7a**, 760 mg, 3.41 mmol, 80% yield) as a yellow oil, which was not purified further. ^{1}H NMR (CDCl$_3$): δ 7.40−7.34 (m, 3H), 7.23 (m, 2H), 5.22 (s, 2H), 2.70 (m, 2H), 2.52 (m, 4H).

To a solution of **7a** (700 mg, 3.14 mmol) in DMF (6.8 mL) in a heavy walled reaction vessel was added sequentially ZnBr$_2$ (1.30 g, 4.98 mmol) and NaN$_3$ (775 mg, 11.9 mmol). The vessel was sealed and heated at 120 °C for 18 h. The resultant mixture was cooled to room temperature and HCl (3 M aq, 2 mL) was added whereupon stirring was continued for 5 min. The mixture was diluted with EtOAc (50 mL) and washed with HCl (1M, aq, 50 mL). The organic portion was dried over $MgSO_4$, the mixture

filtered, and solvent removed under reduced pressure. Purification by silica gel chromatography (50:50:0.2, hexanes:EtOAc:AcOH) gave 1-benzyl-3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (450 mg, 1.69 mmol, 54% yield) as a white solid.

Air was bubbled through a stirring solution of 1-benzyl-3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (450 mg, 1.69 mmol) and KO*t*-Bu (11.8 mL of a 1 M solution in THF) in DMSO (10 mL) for 1.5 h. The reaction mixture was acidified by the addition of 3 M aq HCl (5 mL). The reaction mixture was then adjusted to pH 3 by cautious addition of solid K_2CO_3. The mixture was filtered and solvent removed under reduced pressure. The residue was purified by reverse-phase HPLC to provide 3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole (207 mg, 1.18 mmol, 70% yield) (**5a**) as a white solid. HPLC/MS: (column a) 99%, t_r = 2.28 min, *m/z* (ES+): 177 $[M + H]^+$, 149 $[M-N_2 + H]^+$. ^{1}H NMR (CD_3OD): δ 2.88 (t, 2H, J = 7.0), 2.82 (t, 2H, J = 7.3), 2.64 (quintet, 2H, J = 7.1).

Preparation of 6-Methyl-3-(2H-tetrazol-5-yl)-1,4,5,6-tetrahydrocyclopentapyrazole (5b). Thionyl chloride (5.5 mL, 0.5 M in DMF, 2.75 mmol) was added to a solution of 1-benzyl-6-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carboxylic acid amide (**6b**, 0.467 g, 1.83 mmol) in DMF (17 mL) and the resulting mixture stirred at room temperature for 3 h. $NaHCO_3$ was then added to quench excess thionyl chloride and the solution stirred for an additional 30 min. The mixture was extracted with EtOAc (2 × 30 mL) and the combined extracts washed with brine, dried over $MgSO_4$, filtered, and the solvent removed under reduced pressure to give 1-benzyl-6-methyl-1,4,5,6-tetrahydro-cyclopentapyrazole-3-carbonitrile (**7b**), which was directly without further purification.

Zinc bromide (0.506 g, 2.25 mmol) and sodium azide (0.238 g, 3.66 mmol) were added to a solution of **7b** in DMF (3 mL). The resulting solution was heated under microwave irradiation to 200 °C for 10 min. Additional sodium azide (0.100 g, 1.54 mmol) was added, and the solution was heated to 200 °C for a further 10 min. Solvent was removed under reduced pressure, and the resulting solid was suspended in aqueous 3 M hydrochloric acid and extracted with ethyl acetate (2 × 20 mL). The organic solution was dried over magnesium sulfate and solvent removed under reduced pressure to give 1-benzyl-6-methyl-3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole, which was used directly without further purification. HPLC/MS: (column b) 99%; t_r = 2.43 min; *m/z* (ES+): 281 $[M + H]^+$, 253 $[M-N_2 + H]^+$.

1-Benzyl-6-methyl-3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole was dissolved in formic acid (10% in methanol, 2.5 mL) and palladium black (350 mg) added. The resulting solution was stirred at room temperature for 2 days, filtered, and the residue applied to a 10 g SCX cartridge, which was then eluted with methanol to remove no acidic species. Elution with ammonia (2 M in ethanol) provided 6-methyl-3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydro-cyclopentapyrazole ammonium salt (**5b**) as a white solid (0.111 g, 0.53 mmol). HPLC/MS: (column a) 97%; t_r = 3.03 min; *m/z* (ES+): 191 $[M + H]^+$, 163 $[M-N_2 + H]^+$. ^{1}H NMR (CD_3OD): δ 3.15 (sextet, 1H, J = 7.1, C(6)-H), 2.95–2.85 (m, 1H), 2.82–2.68 (m, 2H), 2.12–2.00 (m, 1H), 1.29 (d, 3H, J = 6.9, CH_3).

Preparation of 5-Methyl-3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydrocyclopentapyrazole (5c). **5c** was prepared using the same method described above for **5b** and the title compound isolated as a white solid (0.053 g, 0.25 mmol). HPLC/MS: (column a) 98%; t_r = 3.08 min; *m/z* (ES+): 191 $[M + H]^+$, 163 $[M-N_2 + H]^+$. ^{1}H NMR (CD_3OD): δ 3.65–3.55 (m, 1H), 3.12–2.85 (m, 2H), 2.41 (ddd, 1H, J_1 = 43.5, J_2 = 14.4, J_3 = 5.2), 1.28–1.13 (m, 3H, CH_3).

Preparation of 4-Methyl-3-(2*H*-tetrazol-5-yl)-1,4,5,6-tetrahydrocyclopentapyrazole (5d). **5d** was prepared using the same method described above for **5b** and the title compound isolated as a white solid (0.032 g, 0.15 mmol). HPLC/MS: (column a) 99%; t_r = 3.30 min; *m/z* (ES+): 191 $[M + H]^+$, 163 $[M-N_2 + H]^+$. ^{1}H NMR (CD_3OD): δ 3.40 (sextet, 1H, J = 6.51, C(4)-H), 2.85–2.60 (m, 3H), 1.26 (d, 3H, J = 6.8, CH_3).

In Vitro Assays. [^{3}H] Nicotinic Acid Binding. Radioligand binding assays were carried out on membranes derived from stably transfected Chinese hamster ovary (CHO) cells. The derivation of the cell lines and the radioligand binding protocol have been described previously.[20]

[^{32}S] GTPγS Binding Assay. [^{32}*S*] GTPγS binding assays were performed as previously described.[20]

Measurement of Adenylyl Cyclase Inhibition. A 96-well adenylyl cyclase activation flashplate assay kit (Perkin-Elmer) protocol was developed and applied as described previously.[22]

Human Subcutaneous Fat Lipolysis Assay. Cultured human subcutaneous adipocytes were received from Zen Bio., Inc. plated in 96-well plates two weeks prior to performing the lipolysis assay. Upon arrival, all media was removed and pooled (ZenBio Adipocyte Maintenance Medium). Then 150 μL of this media was realiquoted to each well. Cells were kept in a sterile, humidified incubator at 37 °C. On the day of the lipolysis assay, cells were washed twice with 150 μL of Zen Bio Wash buffer. After the second wash and removal of wash buffer, 75 μL of test compounds were added to each well in triplicate. Compounds were prepared in Zen Bio Assay buffer plus 1 μM theophylline. Cells were incubated for 5 h at 37 °C. Glycerol was determined using a free glycerol reagent from Sigma (reagent A). Adipocyte media (50 μL) was removed and transferred to a flat-bottom 96-well plate. Reagent A (50 μL) was then added to each well. After 15 min, absorbance was read at OD_{540} on a Spectramax 340PC microplate reader (Molecular Devices). The amount of glycerol released was calculated based on regression analysis of known glycerol concentrations using a Glycerol Standard (Sigma).

In Vivo Assays. Animals. Animal studies were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (1996) and approved by the Arena Pharmaceuticals and Merck Research Laboratories Animal Care and Use Committees. All mice used were C57Bl/6 males, at least 8 weeks old (The Jackson Laboratories West, Sacramento, CA). Animals were housed under standard laboratory conditions with a 12 h dark, 12 h light cycle with constant temperature and humidity. Mice were fed a standard rodent diet, and had ad libitum access to food and water.

In Vivo Mouse Lipolysis. Prior to study, mice were fasted for 16 h. Compound or vehicle (0.5% methylcellulose) was administered by oral gavage (po), and animals were euthanized 20 min postdose by CO_2 asphyxiation. Blood was collected via the inferior vena cava, anticoagulated in EDTA, and plasma separated by centrifugation on a tabletop microcentrifuge. Plasma was used for the measurement of FFA, using an enzymatic method (NEFA C Free Fatty-Acid Assay; WAKO Chemicals USA, Richmond, VA) and for the measurement of compound levels, by API-4000 LC-MS/MS after acetonitrile precipitation.

In Vivo Mouse Vasodilation. Mouse vasodilation was measured by laser Doppler flowmetry as previously described.[22] Briefly, male C57/Bl6 mice (8–10 weeks old; ~25 g) were anesthetized with Nembutal via ip injection (80 mg/10 mL/kg). After 10 min, the mouse was placed under an LDPI laser Doppler (PeriScan PIM II; Perimed, Stockholm) and a needle and syringe containing vehicle (PBS; 40% hydroxypropyl-β-cyclodextrin (HPBCD) or 0.5% methylcellulose) or drug was placed in the intraperitoneal space and a slight back pressure was applied to prevent premature delivery of compound. The mouse's right ear was turned inside-out to expose the ventral side using forceps. The laser Doppler was focused in the center of the ventral right ear and adjusted as follows: repeated data collection; 15 × 15 image format, auto interval start, 20 s delay, medium resolution, very fast scan speed, and 8–9 V intensity (~4.5 cm from ear). After a three minute baseline reading, vehicle or compound was administered into the ip space (5 mL/kg through the preinserted syringe) and readings continued for approximately 15 min. Vasodilation was expressed as "% change of perfusion over baseline values". At the end of the studies, mice were euthanized and a blood sample was collected by cardiac puncture and anticoagulated in EDTA. Plasma was obtained by centrifugation and used for determination of compound concentration by LC-MS/MS.

Supporting Information Available: HPLC-MS spectra, dose-response curves for **5a**, **5a** antagonizes nicotinic acid, PK-PD relationship for **5a** and nicotinic acid in c57/bl6 mice, effect of **5a** and nicotinic acid in PUMA-G knockout mice. This material is available free of charge via the Internet at http://pubs.acs.org.

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Lipidology

Effects of a niacin receptor partial agonist, MK-0354, on plasma free fatty acids, lipids, and cutaneous flushing in humans

Eseng Lai, MD, PhD,* M. Gerard Waters, PhD,† James R. Tata, PhD, Waldemar Radziszewski, MD, PhD, Inna Perevozskaya, PhD, Wei Zheng, MS, Larissa Wenning, PhD, Daniel T. Connolly, PhD, Graeme Semple, PhD, Amy O. Johnson-Levonas, PhD, John A. Wagner, MD, Yale Mitchel, MD, John F. Paolini, MD, PhD

Merck & Co., Inc., 126 East Lincoln Avenue, PO Box 2000, Rahway, NJ 07065-0900, USA (Drs. Lai, Waters, Tata, Radziszewski, Perevozskaya, Zheng, Wenning, Johnson-Levonas, Wagner, Mitchel, and Paolini) and Arena Pharmaceuticals, San Diego, CA, USA (Drs. Connolly and Semple)

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Partial agonist

BACKGROUND: Development of niacin-like agents that favorably affect lipids with an improved flushing profile would be beneficial.

OBJECTIVE: To evaluate a niacin receptor partial agonist, MK-0354, in Phase I and II studies.

METHODS: The pharmacokinetic/pharmacodynamic effects of single and multiple doses (7 days) of MK-0354 (300–4000 mg) were evaluated in two Phase I studies conducted in healthy men. A Phase II study assessed the effects of MK-0354 2.5 g once daily on lipids during 4 weeks in 66 dyslipidemic patients.

RESULTS: MK-0354 single doses up to 4000 mg and multiple doses (7 days) up to 3600 mg produced robust dose-related reductions in free fatty acid (FFA) over 5 hours. Single doses of MK-0354 300 mg and extended release-niacin (Niaspan) 1 g produced comparable reductions in FFA. Suppression of FFA following 7 daily doses of MK-0354 was similar to that after a single dose. In the Phase II study, MK-0354 2.5 g produced little flushing but no clinically meaningful effects on lipids (placebo-adjusted percent change: high-density lipoprotein cholesterol, 0.4%, 95% confidence interval −5.2 to 6.0; low-density lipoprotein cholesterol, −9.8%, 95% confidence interval −16.8 to −2.7; triglyceride, −5.8%, 95% confidence interval −22.6 to 11.9).

CONCLUSION: Treatment with MK-0354 for 7 days resulted in plasma FFA suppression with minimal cutaneous flushing. However, 4 weeks of treatment with MK-0354 failed to produce changes in high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, or triglycerides.

Niacin (nicotinic acid) is an effective treatment for managing multiple lipid/lipoprotein parameters associated with increased cardiovascular risk. At doses >1 g, niacin reduces low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) levels and is the most effective therapy for raising high-density lipoprotein cholesterol (HDL-C).[1] Niacin, administered alone or in combination with other lipid-modifying therapies (statins, bile acid resins, or both), reduces atherosclerotic coronary heart disease (CHD) in dyslipidemic patients with cardiovascular disease[2–5] and may reduce overall mortality.[6] Niacin doses that produce substantial

* Corresponding author.
E-mail address: eseng_lai@merck.com
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†Present address: Global Alliance for TB Drug Development, New York, NY.

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lipid-modifying efficacy (>1 g/day) are frequently associated with bothersome side effects.[7] Flushing of the face, neck, and trunk occurs in most patients (>90%) receiving niacin therapy.[8] These adverse cutaneous reactions limit patient acceptance and have precluded widespread use of niacin.

A G_i-protein-coupled receptor (GPR109A) that binds niacin has been identified on membranes of adipocytes, macrophages, and Langerhans cells in the skin.[9–11] In adipose tissue, niacin-induced activation of GPR109A mediates a potent antilipolytic response by decreasing hydrolysis of TG stores, which in turn reduces plasma levels of free fatty acids (FFA).[11] This effect of niacin on FFA mobilization is postulated to decrease hepatic synthesis of TG, leading to reduced production of very-low-density lipoprotein (VLDL) and, in turn, LDL particles.[12,13] Recent data using mice deficient in GPR109A indicates that niacin-induced cutaneous vasodilation (a model for flushing in humans) is also mediated by GPR109A.[9] Flushing is caused by agonism of niacin receptors on Langerhans cells, which causes the release of arachidonic acid from membrane phospholipids and its subsequent metabolism to prostaglandin D_2 (PGD_2). PGD_2 activates one of its cognate receptors, termed *DP1*, presumably in dermal blood vessels, resulting in cutaneous vasodilation and flushing.[9,14]

Partial agonists of human GPR109A represent a potential novel approach to the treatment of dyslipidemia.[15] Some partial agonists bind their target receptors but stimulate only a subset of downstream signaling pathways.[16,17] MK-0354 is a GPR109A partial agonist that activates the antilipolytic pathway in adipocytes, but does not stimulate ERK 1/2[18] in recombinant cells, a behavior that correlates with reduced flushing potential in preclinical models. This report describes results from three studies designed to evaluate the therapeutic potential of MK-0354 in the treatment of patients with dyslipidemia. The single-dose and multiple-dose pharmacokinetics and pharmacodynamics, as well as tolerability, of MK-0354 were examined in two Phase I studies conducted in healthy male volunteers. The lipid efficacy of MK-0354 was assessed in a Phase II study conducted in male and female patients with dyslipidemia.

Methods

Phase I studies

Two Phase I studies were conducted to evaluate the pharmacokinetic, pharmacodynamic, and safety/tolerability profile of MK-0354. The single-dose study was a randomized, double-blind, placebo-controlled, alternating two-panel, five-period, increasing-dose study conducted in 16 healthy male volunteers. Subjects were assigned to receive single oral doses of placebo (n = 2) or MK-0354 (n = 6) within Panels A (placebo or MK-0354 25, 150, 600, 2400 fasted, and MK-0354 600 mg fed) or B (placebo or MK-0354 75, 300, 1200, 4000 mg fasted, and Niaspan 1 g fasted; Abbott Laboratories, Abbott Park, IL) with ≥7-day washout between doses. In Panel B, blood samples were collected pre-dose and up to 5 hours post-dose for measurement of FFA concentrations.

The multiple dose study was a randomized, double-blind, placebo-controlled, increasing-dose study conducted in 46 healthy male volunteers. Subjects were assigned to receive multiple (7 days) oral doses of placebo (n = 2) or MK-0354 (n = 6) within Panels A (once-daily MK-0354 900 mg), B (once-daily MK-0354 1800 mg), C (once-daily MK-0354 3600 mg) fasted, and Panel D (1800 mg twice daily) administered in the morning fasted and evening following a meal (with no evening dose on day 7). In Panels E and F, one subject received placebo and six subjects received MK-0354 (1800 mg twice daily) for 7 days. In contrast to Panel D, in Panels E and F the morning dose was administered after a standard breakfast and in these panels plasma FFAs were not measured. In Panels A to D, blood samples were collected pre-dose (day 1) and at selected time points up to 6 hours post-dose on day 7 for measurement of FFA concentrations.

Phase II study

The Phase II, multicenter, randomized, double-blind, placebo-controlled study enrolled 66 male and female patients aged 18 to 75 years with dyslipidemia and not on lipid-modifying therapy. Patients with HDL-C ≤70 mg/dL and TG ≥75 and ≤350 mg/dL at the screening visit (ie, visit 1) were eligible for enrollment. Those with calculated creatinine clearance of <80 mL/min were excluded. Additional qualifying criteria (visit 1) included LDL-C ≥125 mg/dL in patients with 0 to 1 risk factor as defined by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) criteria, or LDL-C ≥125 and ≤190 mg/dL in patients with multiple risk factors for CHD. Patients at high risk of CHD were excluded, except for patients with type 2 diabetes without cardiovascular or peripheral vascular disease with LDL-C ≥125 and ≤160 mg/dL (visit 1). Following a 2-week placebo run-in period, patients were randomized to double-blind MK-0354 2500 mg or placebo once daily for 4 weeks. Clinic visits occurred every 2 weeks with a post-study telephone contact (2 weeks after study end). Blood specimens were obtained at each clinic visit for efficacy and safety measurements. Plasma HDL-C, LDL-C, and TG were assayed at baseline, week 2, and week 4. A previously validated Flushing Symptom Questionnaire[19] was completed daily at 8 to 12 hours post-dose during the second week of the placebo run-in period (days −7 to −1), the first 7 days of active treatment (days 1 to 7), and the last 7 days of active treatment (days 22 to 28). One of the Flushing Symptom Questionnaire questions assessed the intensity of all four flushing symptoms (skin redness, warmth, tingling, and/or itching) in aggregate using an 11-point numerical rating scale, the Global Flushing Severity Score (GFSS), which was labeled with the follow-

ing intensity categories: none (score of 0), mild (1–3), moderate (4–6), severe (7–9), and extreme (10).

The Phase I and II study protocols were reviewed and approved by the appropriate ethics committees/institutional review boards. All participants enrolled in these studies provided written informed consent. The study was performed under the guidelines established by the Declaration of Helsinki and Good Clinical Practice standards.

Statistical analyses

Pharmacokinetics. Terminal half-life ($t_{½}$) was calculated as ln(2)/apparent terminal rate constant (λ). The area under the plasma concentration-time curve (AUC) was calculated to 24 hours (AUC_{0-24} [μM · h]) or to the last time point. $AUC_{0-\infty}$ [μM · h] was estimated as the sum of AUC to the last measured concentration and the extrapolated area. Peak plasma concentration (C_{max} [μM]) and its time of occurrence (T_{max} [hours]), as well as peak plasma concentration at 12 (C_{12h}) and 24 hours (C_{24h}), were obtained by inspection of the plasma concentration-time curves.

The pharmacokinetic parameters in the single-dose study were analyzed using an analysis of variance model for an alternating-panel dose-increasing design. The analysis of variance model was a mixed model with fixed effects for panel, treatment-within panel, and random effect subject within panel. The pharmacokinetic parameters in the multiple dose study were analyzed using an analysis of covariance model appropriate for a parallel design. The analysis of covariance model contained terms for dose, baseline body weight, and age. Log transformation was applied to $AUC_{0-\infty}$, AUC_{0-24h}, AUC_{0-12h}, C_{max}, C_{12h}, and C_{24h}. Rank transformation was applied to T_{max} values and an inverse transformation was applied to $t_{½}$.

Efficacy. The effects of MK-0354 on plasma FFA were key secondary end points in the two Phase I studies. This analysis was based on the all-patients–treated (APT) population, which included all patients who took at least one dose of post-randomization study medication, had an FFA measurement at time 0 (prior to dosing) and at least one post-dose FFA measurement on the day of interest. The last observation carried forward method was used to impute missing values, when necessary. Data were presented as logarithm of mean FFA at the specified post-dose time point/baseline FFA (studies 1 and 2). The FFA measurements obtained immediately prior to dosing (ie, time 0) were considered baseline in both studies. The primary pharmacodynamic end point in both studies was the weighted average FFA (ie, $AUC_{(0-4h)}$ for FFA time curve).

For the Phase II study, the primary efficacy analysis was based on the APT population, which included all patients who took at least one dose of post-randomization study medication, had a lipid measurement at baseline, and at least one post-baseline lipid measurement. The last observation carried forward method was used to impute missing values. The placebo-adjusted mean (median for TG) percent changes from baseline to week 4 in HDL-C (primary end point), LDL-C (exploratory end point), and TG (exploratory end point) were determined for MK-0354. An estimation approach was utilized to assess between-group differences involving the construction of two-sided 95% confidence intervals (CIs).

Safety and tolerability. Data from all patients who received at least one dose of study medication were included in safety and tolerability assessments in both studies. The safety of MK-0354 was assessed by clinical evaluation, physical examination, vital signs, and standard fasting laboratory safety tests (hematology, chemistry, and urinalysis). Fasting serum glucose and fasting serum insulin were measured in the Phase IIa study only.

The key prespecified tolerability analysis (secondary end point) focused on the percentage of patients with "severe or greater" flushing symptoms (maximum GFSS ≥7) during week 1 in the Phase II study. The incidences of "moderate or greater" flushing symptoms (maximum GFSS ≥4) were also analyzed as exploratory end points. The analysis population for flushing end points was the APT population who also had at least one respective questionnaire value. There was no imputation of missing data and two-sided 95% CIs were constructed for evaluating between-group differences.

Results

Pharmacokinetics (Phase I studies)

In the single-dose study, MK-0354 was rapidly absorbed with C_{max} occurring within 1 hour after administration of single doses (Fig. 1A). Values for $AUC_{0-\infty}$, C_{max}, C_{12h}, and C_{24h} increased in a dose-related manner across the dose range studied. Values for T_{max} were similar at each dose tested. Food delayed absorption of MK-0354, with a greater effect on the rate than the extent of absorption. Ingestion of a high-fat meal prior to a single 600-mg dose of MK-0354 decreased C_{max} by 60%, increased T_{max} by 1.5 hours, decreased $AUC_{0-\infty}$ by 20%, and increased trough concentrations (C_{12h}) by 50%.

In the multiple-dose study, the pharmacokinetic parameters of MK-0354 increased in a dose-dependent manner following administration of single (day 1) and multiple doses (day 7) ranging from 900 to 3600 mg (Fig. 1B). Overall, the pharmacokinetic profiles for the once-daily dosing regimens were similar on days 1 and 7, indicating there was minimal accumulation of MK-0354 (Table 1). Minor accumulations in AUC_{0-T} and C_{max} and a roughly two-fold accumulation in trough concentrations (C_{12}) were observed following twice-daily dosing of MK-0354 1800 mg in both the fasted and fed states. Food appeared to reduce plasma C_{max}, but had minimal effects on AUC and trough (C_{12h}) levels of MK-0354 when administered as 1800 mg twice daily.

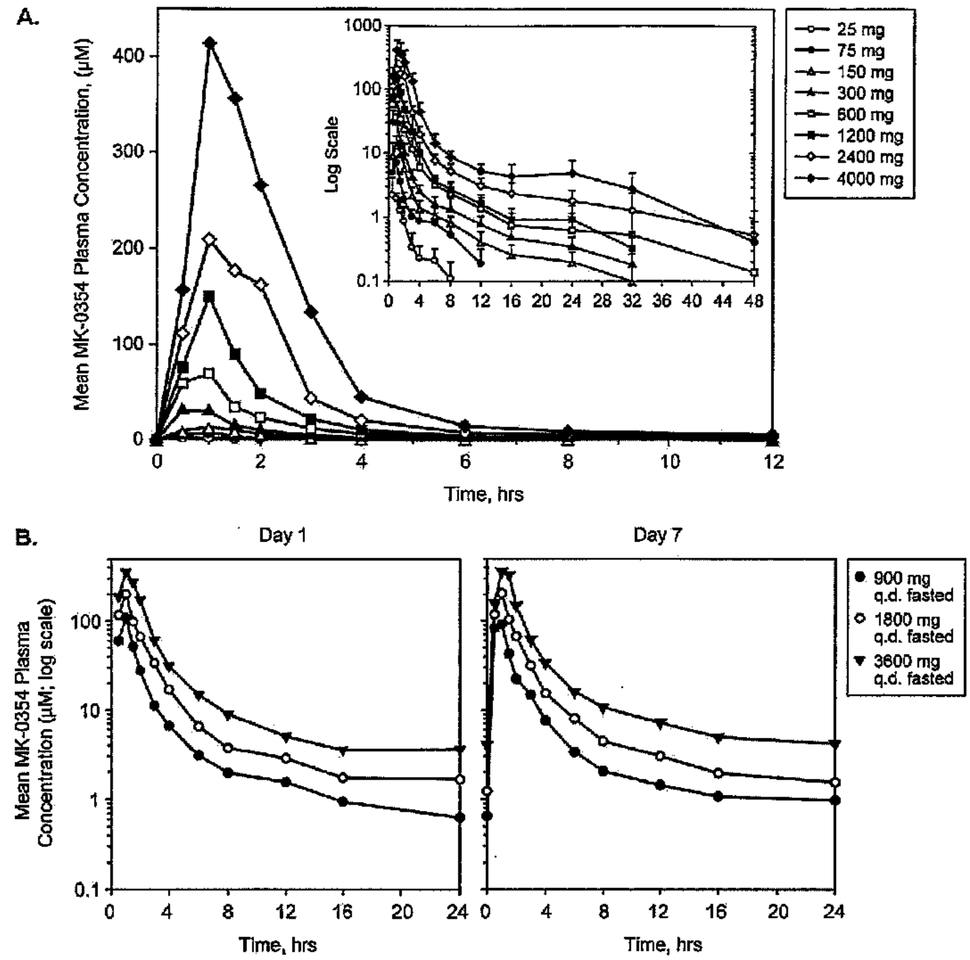

Figure 1 MK-0354 single dose ([A] single-dose study] and multiple dose ([B] multiple-dose study) plasma concentration-time curves in healthy volunteers. Data are plotted as mean MK-0354 plasma concentration (μM) over time (A) and log of mean MK-0354 plasma concentration over time ± standard deviation (inset A, B).

Pharmacodynamic effects on FFA (Phase I studies)

Compared to placebo, single doses of MK-0354 achieved significant dose-dependent decreases in plasma FFA levels over 4 hours post-dose in healthy volunteers (Fig. 2A). The magnitude and duration of the FFA-lowering response induced by MK-0354 for all tested doses was similar to or greater than that seen with Niaspan 1 g. Maximum reductions in FFA were achieved by 1.5 to 2.5 hours (depending on the dose) in the MK-0354 groups and 1 hour post-dose in

Table 1 Mean pharmacokinetic parameters and geometric mean ratios following multiple-dose administration of MK-0354 (multiple-dose Phase I study)

	$AUC_{0\text{-}T}$ (μM · hour)			C_{max} (μM)			C_{12h} (μM)			C_{24h} (μM)			T_{max} (h)†		$T_{1/2}$‡	
Dose (mg)	Day 1	Day 7	Ratio*	Day 1	Day 7	Ratio*	Day 1	Day 7	Ratio*	Day 1	Day 7	Ratio*	Day 1	Day 7	Day 1	Day 7
900 qd	167	173	1.03	114	101	0.89	1.49	1.40	0.93	0.60	0.86	1.44	0.9	0.8	9.0	13.1
1800 qd	356	359	1.01	195	191	0.98	2.61	2.76	1.06	1.35	1.22	0.90	1.0	1.1	10.9	10.2
3600 qd	731	766	1.05	351	373	1.06	4.66	6.30	1.35	3.18	3.63	1.14	1.3	1.2	7.7	9.1
1800 bid	398	487	1.22	228	235	1.03	2.87	6.16	2.15	—	—	—	1.0	0.9	—	9.9
1800 bid fed	319	392	1.23	134	147	1.10	3.34	6.31	1.89	—	—	—	2.0	1.7	—	10.4

AUC, area under the curve; bid, twice daily; qd, once daily; C_{max}, peak plasma concentration; T, length of dosing interval; 24 hours for once-daily dosing and 12 hours for twice-daily dosing; T_{max}, time of occurrence of C_{max}.

*Ratio (day 7/day 1).

†Arithmetic mean.

‡Harmonic mean; day 1 half-life estimated based on data out to 24 hours only.

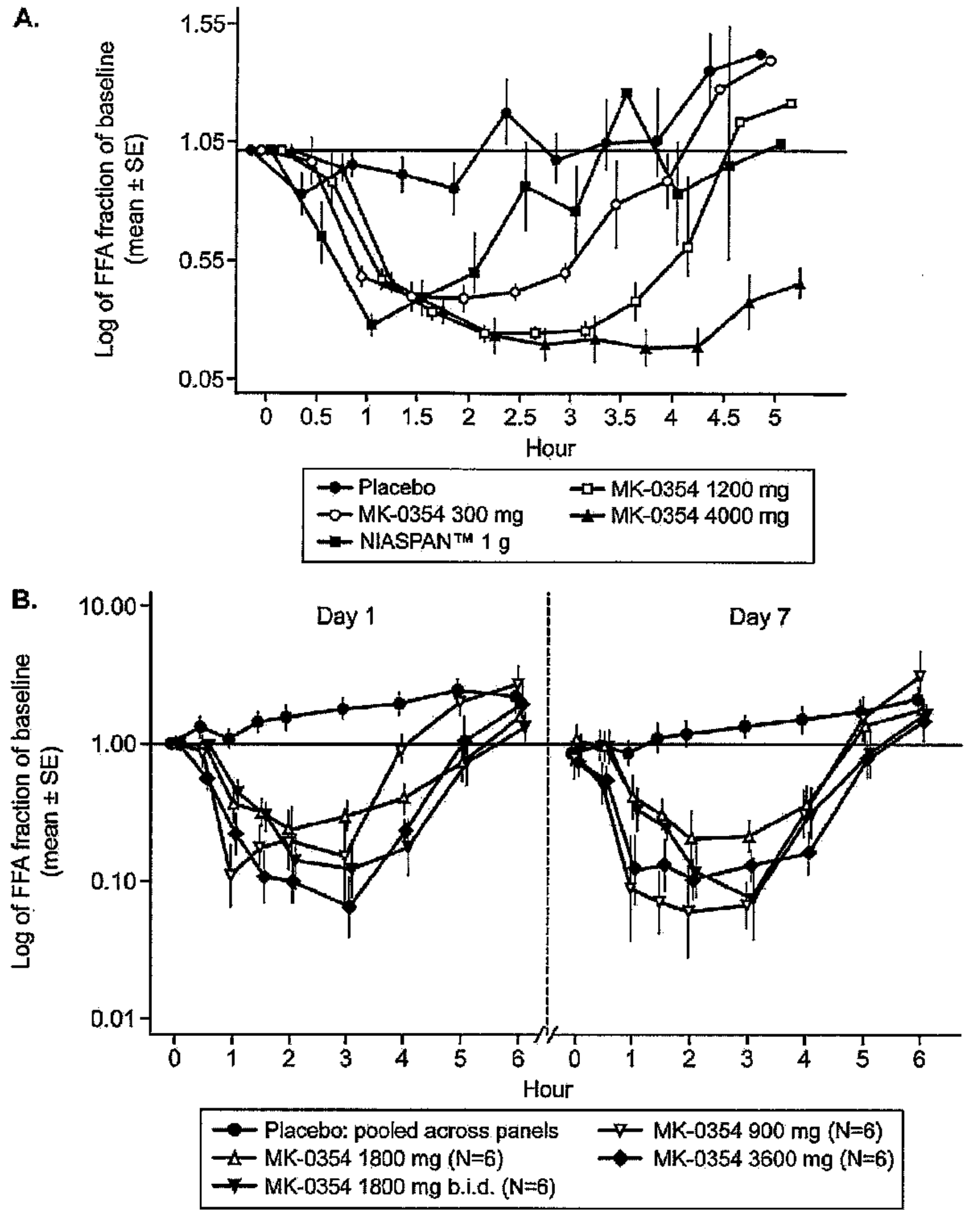

Figure 2 Effects of single (A) and multiple (B) doses of MK-0354 on plasma free fatty acid (FFA) concentrations in healthy volunteers. Results are plotted as log of FFA fraction at baseline (standard error). Blood samples were obtained before dosing (0 hour) and at 30-minute intervals after administration of study drug up to 5 (single-dose study) or 6 hours (multiple-dose study). Data were not carried forward to impute missing values.

the Niaspan group. Peak reduction in FFA observed after administration of single-dose MK-0354 300 mg was comparable to that seen for Niaspan 1 g. At 5 hours post-dose, plasma FFA levels in the MK-0354 300 and 1000 mg groups returned to placebo levels, whereas FFA levels in the 4000 mg group remained markedly depressed. For the single-dose study, the weighted average FFA values (ie, AUC_{0-4h} for the FFA plasma-time curves) for the MK-0354 and Niaspan groups were significantly different than placebo ($P < 0.010$ vs placebo for all groups).

In the multiple-dose study, treatment with single (day 1) and multiple (day 7) once-daily doses of MK-0354 ranging from 900 to 3600 mg led to significant dose-related reductions in FFA (Fig. 2B). For all MK-0354 doses, the FFA-lowering effects were maintained following 7 days of dosing with no evidence of tachyphylaxis. The weighted average FFA values (ie, AUC_{0-4h} for the FFA plasma time curves) for the MK-0354 groups were significantly different than placebo ($P < 0.010$ vs placebo for all groups).

The short sampling durations in these studies precluded a formal evaluation of whether plasma FFA levels rebounded above predose values with MK-0354 treatment, a phenomenon that has been described for niacin.[20]

Lipid efficacy (Phase II study). Of 222 dyslipidemic patients who were screened for entry in the Phase II study, 156

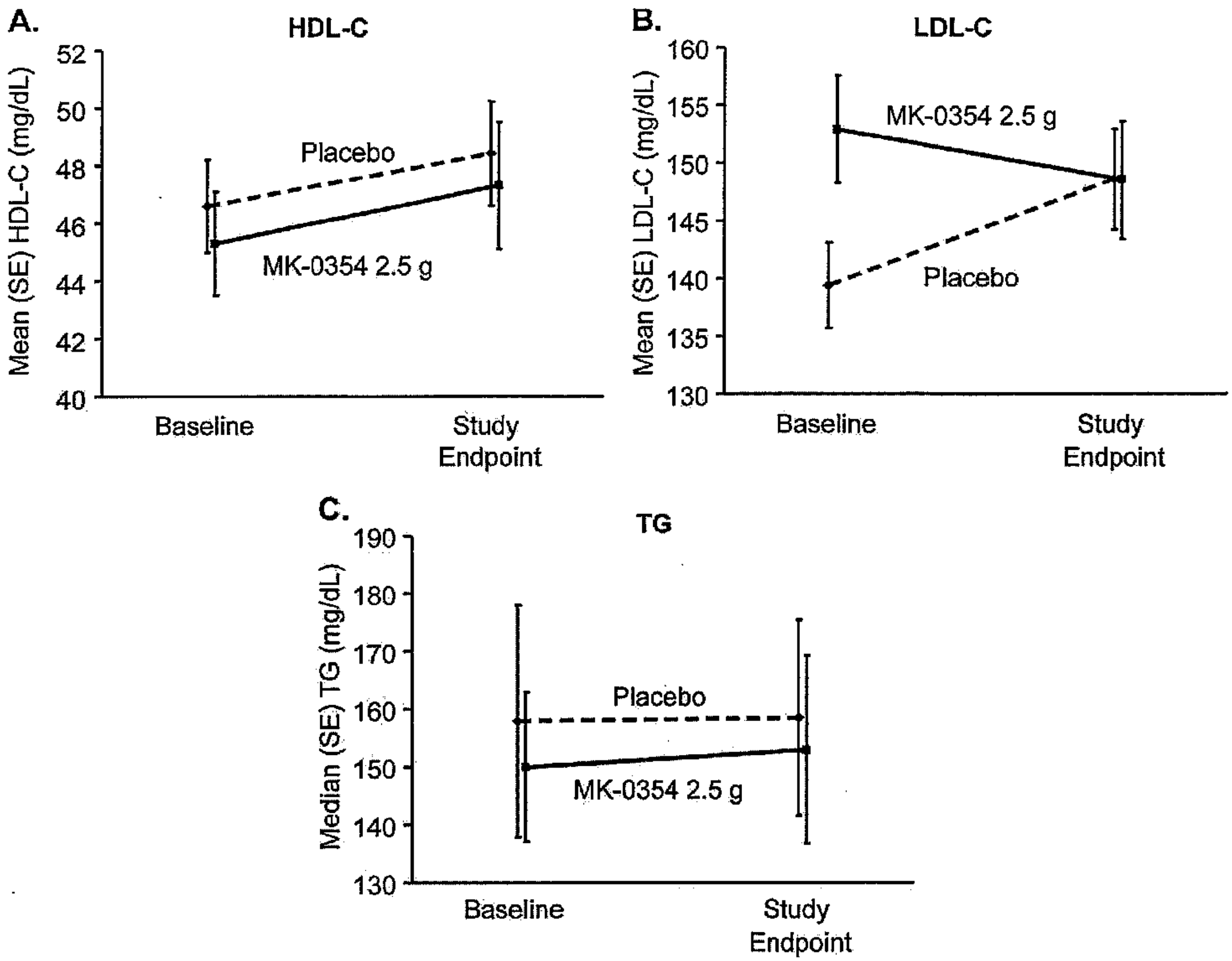

Figure 3 Mean (standard error) plasma concentrations of (A) high-density lipoprotein cholesterol (HDL-C), (B) low-density lipoprotein cholesterol (LDL-C) (C), and median (standard error) plasma concentrations of triglycerides (TG) at baseline and study end point in dyslipidemic patients.

(70.3%) were excluded and 66 (29.7%) were randomized equally to once-daily MK-0354 2500 mg (n = 33) or placebo (n = 33) for 4 weeks. The high screen failure rate in this study was due to patients not meeting the creatinine clearance (≥80 mL/min) and LDL-C (≥125 mg/dL) entry criteria at visit 1. In total, 60 (90.9%) patients successfully completed the entire double-blind treatment phase (n = 31 and n = 29 in the MK-0354 and placebo groups, respectively). There were no clinically meaningful differences between the two groups with regard to the numbers of patients that discontinued or the reasons for discontinuation. Overall, the placebo and MK-0354 groups were well-balanced with respect to patient demographics and baseline HDL-C (mean values were 46.6 and 45.3 mg/dL, respectively) and TG values (median values were 158.0 and 150.0 mg/dL, respectively). There was a slight imbalance between the two groups with respect to baseline LDL-C values (mean values were 139.4 and 152.9 mg/dL, respectively).

Treatment with MK-0354 2500 mg for 4 weeks did not produce clinically meaningful changes in HDL-C, LDL-C, or TG in dyslipidemic patients (Fig. 3). The mean (95% CI) [median (95% CI)] for TG) placebo-adjusted percent change from baseline at study end point for MK-0354 was 0.4% (−5.2 to 6.0), −9.8% (−16.8 to −2.7), and −5.8% (−22.6 to 11.9) for HDL-C, LDL-C, and TG, respectively. Although the placebo adjusted change from baseline for LDL-C was statistically significant −9.8% (−16.8 to −2.7), it was not considered strong evidence of LDL efficacy. Slight imbalances with respect to baseline LDL between treatment groups suggests that this observed (unadjusted) mean between-treatment difference in LDL percent change may be an overestimate of the true treatment effect.

Safety and tolerability (Phase I and II studies). Administration of single and multiple oral doses (7 days) of MK-0354 were well-tolerated in healthy male volunteers. There were no serious adverse events and no discontinuations due to adverse events in the Phase I studies. There also were no clinically significant abnormalities in routine serum chemistry, urinalysis, electrocardiograms, and physical examina-

tions. In the single-dose study, adverse experiences were generally transient in duration and mild-to-moderate in intensity. Flushing symptoms occurred rarely with MK-0354 single 2400 and 4000 mg doses and were mild-to-moderate in intensity, with the exception of two subjects in the 4000-mg group, who reported severe flushing with a rating of "quite bothersome" in the flushing questionnaire. Flushing symptoms with MK-0354 for doses up to 2400 mg were less than for Niaspan 1 g, whereas those for MK-0354 4000 mg were similar to NIASPAN 1 g.

In the Phase II study, MK-0354 was well-tolerated. There were no clinically meaningful differences between the two groups with regard to the incidences or types of adverse experiences. No serious clinical or laboratory adverse events were reported in the study and no patients discontinued treatment due to a clinical or laboratory adverse experience (Table 2). There were no drug-related creatine kinase (CK) elevations in either treatment group. One (3.0%) patient in the MK-0354 group who had an elevated alanine aminotransferase (ALT) value more than one time the upper limit of normal (ULN) upon entering the study (day 1) presented with a single ALT measurement more than three times ULN on day 28. There were no reports of any liver-related adverse events in this study. Treatment with MK-0354 2.5 g led to small mean increases in fasting serum glucose and fasting serum insulin compared to placebo (Table 2). The placebo-adjusted mean increases were 5.6 mg/dL (95% CI, −1.3 to 12.4) and 7.4 μIU/mL (95% CI, −0.3 to15.1) for the fasting serum glucose and fasting serum insulin, respectively. As this study was not powered to definitively assess statistical significance for these two parameters, these findings do not conclusively demonstrate a treatment effect. No patient experienced laboratory adverse experiences of increased fasting plasma glucose or insulin during this study.

There was no significant between-group difference in the percentage of patients reporting moderate or greater (GFSS $\geq$4) or severe or extreme flushing (GFSS $\geq$7) during the initiation phase (ie, week 1) or maintenance phase (ie, week 4) of treatment (Table 2).

Discussion

This is the first published report describing the efficacy and safety/tolerability profile of a partial agonist of the niacin receptor in humans. MK-0354, a pyrazole tetrazole compound, was identified as a therapeutic candidate based on its partial agonist activity at GPR109A in in vitro experiments.[18] MK-0354 was similar to niacin in its activity to inhibit human adipocyte lipolysis (ie, decreased glycerol production in response to isoproterenol in vitro) but, unlike niacin, did not produce signaling events triggered by compounds known to induce flushing (ie, failed to induce niacin receptor internalization and stimulate ERK 1/2 MAP kinase).[18] In animal models, MK-0354 retained full antilipolytic activity with a greatly diminished cutaneous vasodilation response.[18] Based on these data, MK-0354 was advanced to clinical studies to determine whether this compound (1) could inhibit lipolysis in people without inducing symptoms of flushing and (2) has the beneficial effects of niacin on the lipid profile.

Phase I clinical studies demonstrated that MK-0354 had a favorable pharmacokinetic profile and was generally well-tolerated in healthy volunteers. Dose-related increases in $AUC_{0-\infty}$ (single-dose study), AUC_{0-24h} (multiple-dose study), C_{max}, C_{12h}, and C_{24h} were seen following treatment with single and multiple doses. The steady-state pharmacokinetics of MK-0354 were generally consistent with those observed following administration of single doses in healthy male volunteers.

Administration of MK-0354 single doses up to 4000 mg and multiple doses (7 days) up to 3600 mg produced significant dose-related reductions in plasma FFA. In the single-dose study, treatment with Niaspan 1 g administered in the fasted state produced reductions in plasma FFA comparable to MK-0354 300 mg. The durations of the FFA-lowering effects of 300, 1000, and 4000-mg MK-0354 were similar to or greater than that seen with Niaspan 1000 mg. The durations of the FFA-lowering effects were maintained for at least 4 hours post-dose in the MK-0354 groups compared to 3 hours for Niaspan, suggesting comparable to more prolonged niacin receptor activation for MK-0354 compared to extended-release niacin. In the multiple-dose study, the FFA-lowering response elicited by MK-0354 was comparable between days 1 and 7. There was no evidence of tachyphylaxis in the activity of MK-0354 to inhibit lipolysis during this period.

Robust reductions in FFA were observed in the Phase I studies with doses of MK-0354 that were associated with minimal flushing symptoms. In the Phase II study, relative to placebo, treatment with MK-0354 2500 mg led to minimal flushing during weeks 1 and 4. These findings provide evidence in humans that flushing and FFA-lowering effects of niacin can be selectively modulated, and suggest that they may be mediated by at least partially distinct pathways.[21,22]

The mechanism by which niacin exerts its beneficial effects on plasma lipids is not fully understood, with multiple hypotheses proposed in the literature. One longstanding hypothesis suggests that inhibition of lipolysis within adipose tissue is a key early event. Inhibition of lipolysis is proposed to result in lower plasma TG levels by limiting the amount of FFA substrate available for use in hepatic synthesis of TG and VLDL. The attenuated synthesis of TG-rich VLDL particles may lead to reductions in LDL, as well as a decreased rate in HDL metabolism by limiting the cholesterol ester transfer protein–mediated exchange of cholesterol from HDL to VLDL, and TG from VLDL to HDL.[20,23,24] Other proposed mechanisms for niacin effects independent of the inhibition of lipolysis have been reviewed recently.[25]

Table 2 Summary of safety and tolerability results in Phase II study

	Placebo (n = 33)	MK-0354 2.5 g (n = 33)	Between-group difference (95% CI)
One or more adverse experiences	5 (15.2)	7 (21.2)	NC
Drug adverse experiences*	3 (9.1)	2 (6.1)	NC
Serious adverse experiences	0	0	NC
Deaths	0	0	NC
Discontinued due to adverse experiences	0	0	NC
Consecutive/presumed consecutive ALT and/or elevation ≥3× ULN†	0/32	1/33 (3.0)	NC
CK elevation ≥10× ULN†	0/32	0/33	NC
Mean (SD) change from baseline in fasting serum glucose (mg/dL)	1.7 (13.6)	7.2 (14.1)	5.6 (−1.3 to 12.4)
Mean (SD) change from baseline in fasting serum insulin (μIU/mL)	−0.4 (5.2)	7.0 (20.4)	7.4 (−0.3 to 15.1)
Week 1 (initiation phase)			
Moderate or greater flushing (maximum GFSS ≥4)	2/33 (6.1)	2/33 (6.1)	0 (−14.2 to 14.2)
Severe or extreme flushing (maximum GFSS ≥7)	0/33	1/33 (3.0)	3.0 (−7.7 to 15.3)
Week 4 (maintenance phase)			
Moderate or greater flushing (maximum GFSS ≥4)	2/27 (7.4)	5/30 (16.7)	9.3 (−9.2 to 27.0)
Severe or extreme flushing (maximum GFSS ≥7)	0/27 (0)	0/30 (0)	0 (−12.5 to 11.4)

ALT, alanine amino transferase; CI, confidence interval; CK, creatine kinase; GFSS, Global Flushing Severity Score; NC, not calculated; SD, standard deviation; ULN, upper limit of normal.

*Determined by the investigator to be possibly, probably, or definitely drug-related.

†Number of patients with elevated test value/number of patients tested.

MK-0354 2500 mg was selected for use in the Phase II study based on projections from Phase I data. It was estimated that at this dose of MK-0354, the extent and duration of FFA-lowering would match or exceed that obtained with 2-g Niaspan, which is known to be efficacious and is the maximal clinical dose of that drug (data not shown). Despite robust reductions in FFA seen with MK-0354 at doses of 1200 mg and higher in the Phase I studies, treatment with once-daily MK-0354 2500 mg for 4 weeks failed to reproduce the beneficial lipid-modifying effects of niacin previously observed in dyslipidemic patients.

Based on available data, it is not understood why long-term treatment with MK-0354 failed to produce an altered global lipid profile similar to niacin. It should be noted that the FFA-lowering response of MK-0354 was not evaluated in the Phase II study; thus it is possible that tachyphylaxis occurred during the extended 4-week treatment period. This explanation may be unlikely because there was no evidence for desensitization in the FFA response following 7 consecutive days of treatment in the multiple-dose Phase I study. Alternatively, these results may imply that mechanisms other than GPR109A-mediated FFA suppression contribute to the global lipid effects of niacin, and that MK-0354 is unable to promote these effects. Further studies are needed to resolve these issues and to determine the mechanism(s) underlying niacin's beneficial lipid effects.

Conflict of interest disclosure

The studies reported in this publication were funded by Merck & Co., Inc. Eseng Lai, James R. Tata, Waldemar Radziszewski, Inna Perevozskaya, Wei Zheng, Larissa Wenning, Amy O. Johnson-Levonas, John A. Wagner, Yale Mitchel, and John F. Paolini are employees of Merck & Co., Inc. and may hold stock in the company. M. Gerard Waters is a previous employee of Merck &Co Inc and may hold stock in the company. D. Connolly and G. Semple are employees of Arena Pharmaceuticals and may hold stock in that company.

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PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect

SORIN TUNARU[1], JUKKA KERO[1], ANNETTE SCHAUB[2], CHRISTIAN WUFKA[2,3], ANDREE BLAUKAT[1], KLAUS PFEFFER[2,3] & STEFAN OFFERMANNS[1]

[1]Institute of Pharmacology, University of Heidelberg, Heidelberg, Germany
[2]Institute of Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Munich, Germany
[3]Institute of Medical Microbiology, University of Düsseldorf, Düsseldorf, Germany
S.T., J.K., A.S. & A.B. contributed equally to this study.
Correspondence should be addressed to S.O. or K.P.; e-mail: Stefan.Offermanns@urz.uni-heidelberg.de, Klaus.Pfeffer@uni-duesseldorf.de

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Nicotinic acid (niacin), a vitamin of the B complex, has been used for almost 50 years as a lipid-lowering drug[1,2]. The pharmacological effect of nicotinic acid requires doses that are much higher than those provided by a normal diet[3,4]. Its primary action is to decrease lipolysis in adipose tissue by inhibiting hormone-sensitive triglyceride lipase[5]. This anti-lipolytic effect of nicotinic acid involves the inhibition of cyclic adenosine monophosphate (cAMP) accumulation in adipose tissue[6] through a G_i-protein-mediated inhibition of adenylyl cyclase[7–9]. A G-protein-coupled receptor for nicotinic acid has been proposed in adipocytes[10,11]. Here, we show that the orphan G-protein-coupled receptor, 'protein upregulated in macrophages by interferon-γ' (mouse PUMA-G, human HM74)[12,13], is highly expressed in adipose tissue and is a nicotinic acid receptor. Binding of nicotinic acid to PUMA-G or HM74 results in a G_i-mediated decrease in cAMP levels. In mice lacking PUMA-G, the nicotinic acid–induced decrease in free fatty acid (FFA) and triglyceride plasma levels was abrogated, indicating that PUMA-G mediates the anti-lipolytic and lipid-lowering effects of nicotinic acid *in vivo*. The identification of the nicotinic acid receptor may be useful in the development of new drugs to treat dyslipidemia.

Based on the finding that specific binding sites of nicotinic acid exist on membranes of adipocytes, spleen and macrophages[11,14], we searched for orphan G-protein-coupled receptors expressed in adipose tissue and cells of the immune system. The mouse orphan receptor PUMA-G is encoded by an interferon-γ-inducible gene and is expressed in various tissues, including macrophages and spleen[13]; its human ortholog, HM74, is highly expressed in activated neutrophils[12,15]. We found that both the human and murine orphan receptors are expressed at high levels in white and brown adipose tissue (Figs. 1*a–c*). Expression was also detected in various other tissues, including lung, adrenal gland and spleen (Figs. 1*a–c*). The coding regions of the genes encoding PUMA-G and HM74 (*Pumag* and *HM74*, also known as *GPR109*) both of which are single-exon genes, were amplified by PCR from mouse adipocyte cDNA and human genomic DNA, respectively, and subcloned into a mammalian expression vector.

To test whether PUMA-G and HM74 function as receptors for nicotinic acid, the two genes were each co-transfected, with the gene encoding the promiscuous G-protein α-subunit $G\alpha_{15}$ (ref. 16), in Chinese hamster ovary (CHO)-K1 cells stably expressing a highly sensitive Ca^{2+} reporter consisting of green fluorescent protein fused to aequorin[17]. Upon exposure to nicotinic acid, we detected a concentration-dependent Ca^{2+} mobilization response in cells expressing PUMA-G or HM74 and $G\alpha_{15}$ (Figs. 1*d* and *e*). Nicotinic acid concentrations for half-maximal responses (EC_{50}) were about 3 μM for the mouse and about 1 μM for the human receptor. Nicotinic acid had no effect in untransfected cells (data not shown) or cells transfected with $G\alpha_{15}$ only (Fig. 1*e*). We also used several structural analogs of nicotinic acid to test for Ca^{2+} responses in cells expressing PUMA-G or HM74 and $G\alpha_{15}$. The rank order of their potencies (Figs. 1*d* and *e*; acipimox, EC_{50} 2–5 μM; pyrazine-2-carboxylic acid, EC_{50} 10 μM; furan-3-carboxylic acid, EC_{50} > 100 μM) correlated with their reported potencies in fat cells, as measured by inhibition of adenylyl cyclase or stimulation of guanosine triphosphate (GTP)-γS binding[11,18].

The cellular effects of nicotinic acid may be mediated by pertussis toxin–sensitive G-proteins of the G_i-family[7,19]. To assess whether activation of PUMA-G and HM74 by nicotinic acid induces G_i-mediated signaling events, we measured inhibition of adenylyl cyclase and activation of extracellular signal–regulated kinase (ERK; see Supplementary Methods online). In cells expressing PUMA-G or HM74 with β_2-adrenergic receptor, nicotinic acid decreased intracellular cAMP, which was raised by the β-adrenergic receptor agonist isoproterenol, in a concentration-dependent manner (Fig. 2*a*). The inhibition of adenylyl cyclase by PUMA-G and HM74 could be completely blocked by pretreating cells with pertussis toxin (PTX; Fig. 2*a*). Activation of ERK by PUMA-G (Fig. 2*b*) or HM74 (data not shown) was also sensitive to PTX. Nicotinic acid had no effects on cAMP concentrations or ERK phosphorylation in untransfected cells. PUMA-G or HM74 did not mediate nicotinic acid–dependent production of inositol phosphates (data not shown). Thus, PUMA-G and HM74 are coupled to G_i-type G-proteins. This confirms studies showing that nicotinic acid–induced anti-lipolytic effects are sensitive to PTX[20].

We then performed radioligand binding assays using ^{3}H-labeled nicotinic acid. Saturation binding analysis showed that membranes prepared from HEK-293 cells transfected with PUMA-G and HM74 showed saturable and specific binding (Figs. 2*c* and *d*; dissociation constant (K_d) was 83.3 nM for PUMA-G and 55.6 nM for HM74). Competitive binding assays (Fig. 2*e*) with differ-

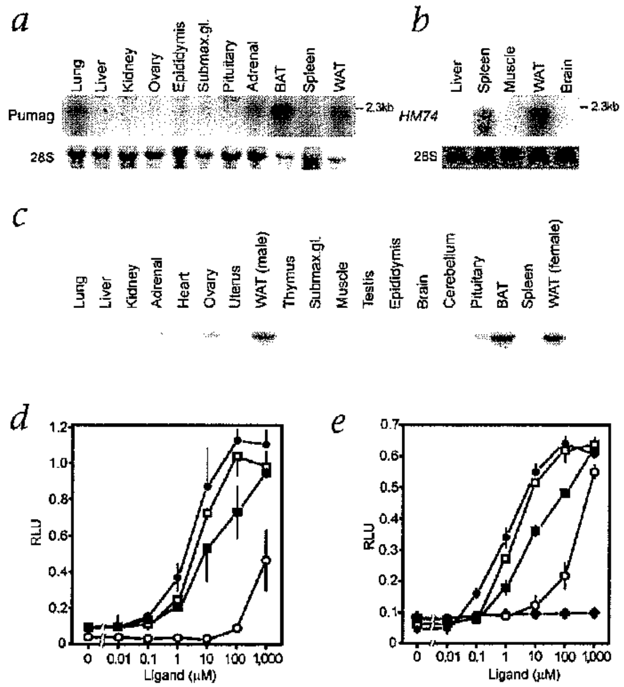


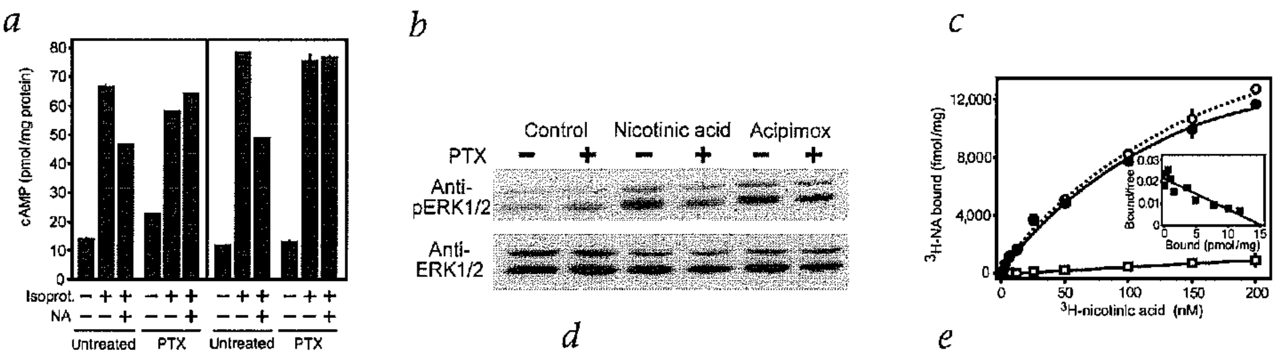
Fig. 1 PUMA-G and HM74 expression in adipose tissue. ***a*** and ***b***, Northern blot analysis of *Pumag* mRNA (*a*) and *HM74* mRNA (*b*) in mouse and human tissues. 28S, ribosomal RNA used as control. ***c***, RT-PCR on cDNAs from different mouse tissues using *Pumag*-specific primers. BAT, brown adipose tissue; WAT, white adipose tissue; submax. gl., submaxillary gland. ***d*** and ***e***, Concentration response relationships of the $[Ca^{2+}]_i$ changes evoked in CHO-K1 cells expressing PUMA-G and $G\alpha_{15}$ (*d*) or HM74 and $G\alpha_{15}$ (*e*) by nicotinic acid (●), acipimox (□), pyrazine-2-carboxylic acid (■) and furan-3-carboxylic acid (O). ◆, indicate values obtained using nicotinic acid in cells expressing only $G\alpha_{15}$. RLU, relative light units. Data are mean ± s.e.m; n=3

ent nicotinic acid analogs showed a rank order of affinities similar to that described for the endogenous binding site[11].

Maximal lipid-lowering effects of nicotinic acid can be seen at plasma concentrations of 4–16 μM[3,21], and peak plasma concentrations after administration of a pharmacological standard dose are in the range of 50–300 μM[3,22]. Thus, the EC_{50} values for nicotinic acid–induced intracellular Ca^{2+} elevation through PUMA-G or HM74 and $G\alpha_{15}$, and the binding affinity of nicotinic acid for PUMA-G and HM74, confirm that PUMA-G and HM74 have a role in the pharmacological effect of nicotinic acid *in vivo*.

The anti-lipolytic effect of nicotinic acid is a result of the inhibition of hormone-sensitive lipase in adipocytes. We used PUMA-G-deficient mice generated by homologous recombination (Fig. 3*a* and *b*) to test whether this anti-lipolytic effect is mediated by PUMA-G. Mice homozygous for the targeted allele of the *Pumag* gene are viable and have no obvious phenotypic abnormalities. The high-affinity binding capacity for nicotinic acid was reduced by >75% in adipocyte plasma membranes from PUMA-G-deficient mice, compared to membranes from wild-type mice (Fig. 3*c*). In addition, the nicotinic acid–induced inhibition of FFA release from isolated adipocytes was completely absent from PUMA-G-deficient fat cells (Fig. 3*d*). This indicates that the metabolic effects of nicotinic acid in adipocytes are indeed mediated by PUMA-G and HM74.

To study the role of PUMA-G in the anti-lipolytic and lipid-lowering effects of nicotinic acid *in vivo*, we compared FFA levels in wild-type mice and PUMA-G-deficient mice after 24 h of starvation. Wild-type mice had higher basal levels of FFAs than did PUMA-G-deficient animals. Within 15 min, intraperitoneal injection of nicotinic acid resulted in a strong decrease in plasma



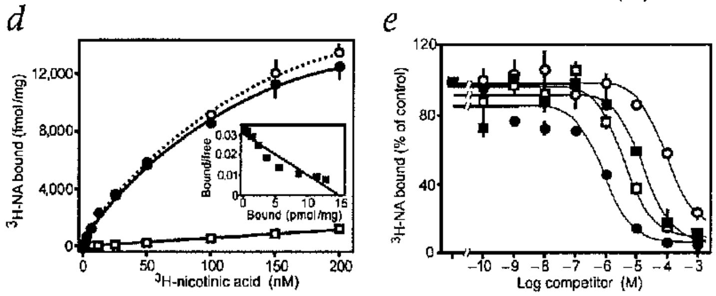


Fig. 2 PUMA-G and HM74 are G_i-coupled receptors for nicotinic acid and related compounds. ***a***, Effect of nicotinic acid (NA; 200 μM) on intracellular cAMP in untreated or PTX-treated (100 ng/ml overnight) CHO-K1 cells expressing the β_2-adrenergic receptor (β_2-AR) and PUMA-G (left) or HM74 (right) after exposure to isoproterenol (isoprot.; 1 μM). ***b***, Effect of PTX on ERK1/2 phosphorylation induced by 200 μM of nicotinic acid or acipimox in PUMA-G-expressing CHO-K1 cells. Shown is 1 representative experiment of 3. ***c*** and ***d***, Saturation binding isotherms for ^{3}H-labeled nicotinic acid in cell membranes from HEK-293 cells transfected with PUMA-G (*c*) and HM74 (*d*). O, total binding; □, nonspecific binding; ●, specific binding. Insets: Scatchard analyses of ^{3}H-nicotinic acid binding saturation isotherm. ***e***, Competitive binding analysis of PUMA-G with nicotinic acid (●), acipimox (□), pyrazine-2-carboxylic acid (■) and furan-3-carboxylic acid (O). Results shown are the mean ± s.e.m. of triplicate determinations.

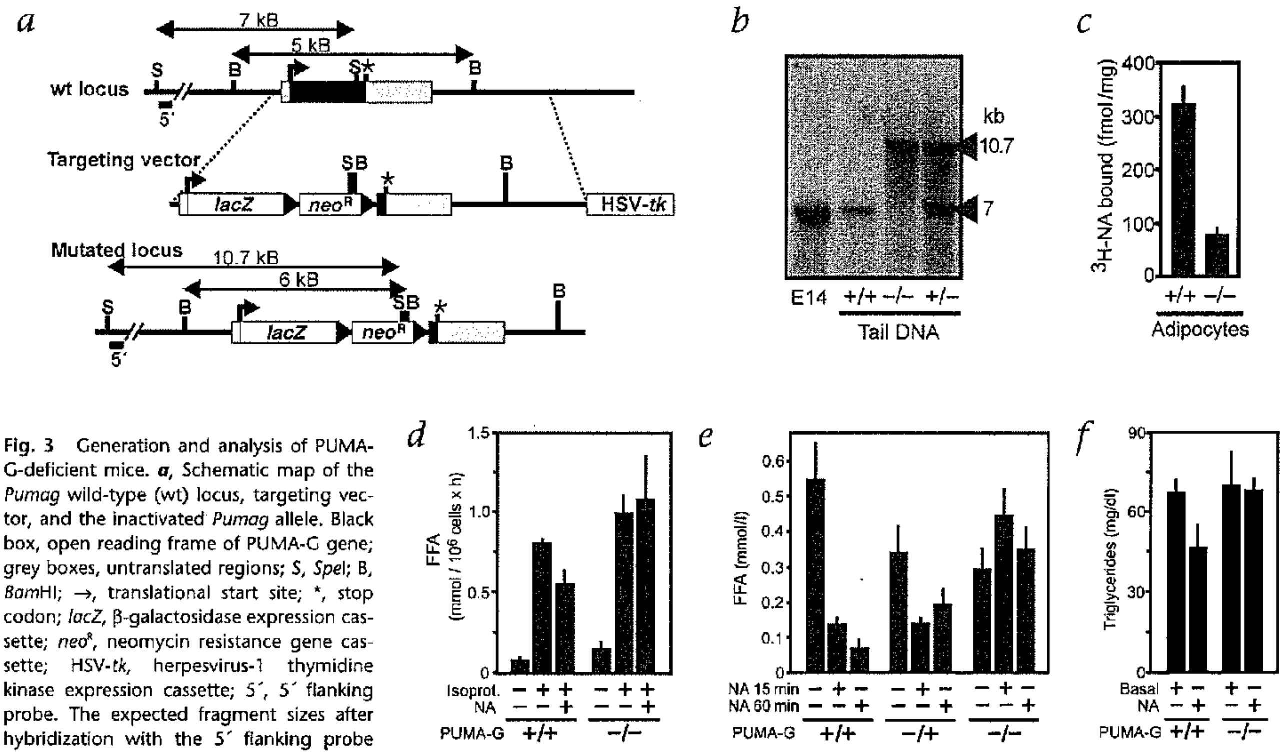


Fig. 3 Generation and analysis of PUMA-G-deficient mice. ***a***, Schematic map of the *Pumag* wild-type (wt) locus, targeting vector, and the inactivated *Pumag* allele. Black box, open reading frame of PUMA-G gene; grey boxes, untranslated regions; *S*, *Spe*I; B, *Bam*HI; →, translational start site; *, stop codon; *lacZ*, β-galactosidase expression cassette; *neo*^{R}, neomycin resistance gene cassette; HSV-*tk*, herpesvirus-1 thymidine kinase expression cassette; 5′, 5′ flanking probe. The expected fragment sizes after hybridization with the 5′ flanking probe (*Spe*I digest of genomic DNA) are indicated. ***b***, Southern blot analysis of mice after germline transmission of the *Pumag* mutation. Hybridization of *Spe*I-digested tail DNA of representative mice is shown (E14, control DNA from E14.1 embryonic stem cells). ***c***, Equilibrium binding of 100 nM of ^{3}H-labeled nicotinic acid to membranes of wild-type and PUMA-G-deficient adipocytes. Nonspecific binding was determined in the presence of 100 μM unlabeled nicotinic acid. ***d***, FFA release from wild-type (+/+) and PUMA-G-deficient (−/−) adipocytes, in the absence or presence of isoproterenol (isoprot.; 1 μM) or isoproterenol and nicotinic acid (NA; 100 μM). ***e***, Plasma FFA concentrations before and 15 or 60 min after injection of nicotinic acid (NA) in wild-type (+/+), heterozygous (−/+) and PUMA-G-deficient mice (−/−). ***f***, Plasma triglyceride levels in wild-type (+/+) and PUMA-G-deficient mice (−/−) before (basal) and after 2 weeks of treatment with nicotinic acid (NA). Results shown are mean ± s.e.m. of 3–5 independent experiments.

levels of FFAs in wild-type mice and mice heterozygous for the targeted PUMA-G allele; the effect lasted >1 h (Fig. 3*e*). In contrast, mice lacking PUMA-G did not show any decrease in FFAs after injection with nicotinic acid. There was a slight increase in FFAs after 15 min, which was also seen in control mice that received only the carrier solution (data not shown). To test whether PUMA-G is involved in nicotinic acid–induced decrease in triglycerides, we administered nicotinic acid to wild-type mice and PUMA-G-deficient mice that were kept on a high-fat diet for 2 weeks. This treatment resulted in a decrease in triglycerides of about 30% in wild-type animals, but had no effect in the absence of PUMA-G (Fig. 3*f*).

Our data clearly show that PUMA-G and HM74 mediate the main metabolic effects of nicotinic acid. It is, however, unlikely that nicotinic acid is the physiological ligand of PUMA-G and HM74. Plasma and serum concentrations of nicotinic acid are mainly determined by diet and are in the range of 100–400 nM[22,23], a concentration that is hardly able to activate PUMA-G and HM74 (Fig. 1*d* and *e*). Among the receptors most similar to PUMA-G and HM74 are peptide receptors, purinoceptors and receptors that respond to lipids, such as the recently described receptor for 5-oxo-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (5-oxo-ETE)[24]. 5-Oxo-ETE has no agonistic activity against PUMA-G or HM74 (data not shown). The orphan receptor GPR81, which has the highest similarity to PUMA-G and HM74 (44% amino acid identity), is not a receptor for nicotinic acid (data not shown). The identity and physiological function of the endogenous ligand of PUMA-G and HM74 are not yet clear.

In conclusion, we have identified a receptor for the anti-dyslipidemic drug nicotinic acid. We have also shown that the receptor for nicotinic acid is highly expressed in white and brown adipose tissue, which is consistent with a role in lipid metabolism. Our studies of mice lacking the nicotinic acid receptor indicate that it is the main mediator of the anti-lipolytic and lipid-lowering effects of nicotinic acid *in vivo*. The identification of the nicotinic acid receptor should encourage further research into its physiological function and may be helpful in the development of anti-dyslipidemic drugs.

Methods

Molecular cloning. *Pumag* and *HM74* were cloned by PCR from white adipose tissue cDNA and genomic DNA, respectively, using primers flanking the full-length coding sequence, and were inserted into the vector pcDNA3.1 (Invitrogen, Carlsbad, California).

Northern blot analysis and RT-PCR. Total RNA (15 μg) from mouse or human tissues (BioCat, Heidelberg, Germany) was resolved on 1% denaturing agarose gels and transferred onto nylon membranes (Amersham Biosciences, Piscataway, New Jersey). After prehybridization, membranes were incubated with a cDNA probe (whole coding region; specific activity >1 × 10^9 cpm/μg) overnight. After washing the membranes, the hybridized probe was visualized by autoradiography. For RT-PCR, 1 μg of total RNA was reverse transcribed, and *Pumag* cDNA was amplified with primers

flanking the full-length coding sequence. A 395-bp fragment of the gene encoding the L19 ribosomal protein was co-amplified as a control.

Calcium mobilization. CHO-K1 cells stably transfected with a calcium-sensitive bioluminescent fusion protein consisting of aequorin and green fluorescent protein[17] were seeded in 96-well plates and transfected with indicated cDNAs or control DNA (50 ng/well) using FuGENE6 reagent (Roche Diagnostics, Indianapolis, Indiana). Two days after transfection, cells were loaded with 5 μM of coelenterazine *h* (Biotium, Hayward, California) in calcium-free Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES (pH 7.4) and incubated for 3.5 h at 37 °C. The buffer was replaced with HBSS containing 1.8 mM $CaCl_2$, 45 min before experiments. Measurements were taken using a luminometer plate reader (Luminoskan Ascent, Labsystems Helsinki, Finland). Nicotinic acid (pyridine-3-carboxylic acid), pyrazine-2-carboxylic acid and furan-3-carboxylic acid were from Sigma (St. Louis, Missouri); acipimox (5-methylpyrazine-2-carboxylic acid 4-oxide) was from Pharmacia-Upjohn (Peapack, New Jersey).

Radioligand binding. Equilibrium binding of ^{3}H-labeled nicotinic acid (50 Ci/mmol; American Radiolabeled Chemicals, St. Louis, Missouri) was done using 30 μg of membranes from HEK-293 cells expressing PUMA-G or HM74 receptors, in a total volume of 250 μl binding buffer (50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$ and 0.02% (v/v) CHAPS). After 4 h of incubation at 25 °C, unbound and membrane-bound radioactivity were separated by filtration of the samples through nitrocellulose filters, followed by two washing steps with 4 ml ice-cold binding buffer. Nonspecific binding was determined in the presence of 200 μM unlabeled nicotinic acid. Competitive binding analysis was done in the presence of 60 nM ^{3}H-nicotinic acid. ^{3}H-nicotinic acid binding to adipocyte membranes was determined using 75 μg of membranes.

Generation of PUMA-G-deficient mice. A 5-kb *Pumag* genomic clone derived from a bacterial artificial clone (Genome Systems, St. Louis, Missouri) was sequenced (GenBank accession no. AJ300199; see Supplementary Methods online). Genotyping of the *Pumag* alleles was performed by PCR using the primers PUMA-G-sense-1 (5′-TCAGATCTGACTCGTCCACC-3′) and 333-KO (5′-CCTCTTCGCTATTACGCCAGC-3′) for the inactivated allele and the primers PUMA-G-sense-1 and 333-WT (5′-CCATTGCCCAGGAGTCCGAAC-3′) for the wild-type allele. Heterozygous mice were back-crossed 5 times into the C576BL/6 strain, and homozygous offspring were obtained by intercrossing *Pumag*$^{+/-}$ mice. Animals were housed in a specified pathogen-free animal facility.

Determination of FFA and triglyceride levels. Each experiment was conducted with 1–3 mice per group, using wild-type littermates as controls. For determination of FFA levels, mice were fed with commercial mouse chow and tap water *ad libitum*. Animals were starved for 24 h before the experiment. Anaesthesia was induced with intraperitoneally injected xylazine hydrochloride (3 mg per kg body weight) and ketamine hydrochloride (100 mg per kg body weight). Blood samples were taken from the retrobulbar capillary plexus and a dose of nicotinic acid at 200 μmol per kg body weight (150 μl) or an equal volume of carrier (0.9% NaCl) was injected intraperitoneally. Blood was collected into heparinized tubes and transferred rapidly onto ice. Plasma was separated by centrifugation and plasma FFA levels were determined by an enzymatic colorimetric method (Roche Diagnostics). For *in vitro* measurements of FFA release from adipocytes, cells were isolated from epididymal, perirenal and mesenterial fat deposits[25]. For plasma triglyceride determinations, mice were kept on a high-fat diet and received 20% sucrose with their drinking water. Blood samples were collected at 11 a.m. before and 2 weeks after treating animals with 2 daily doses of nicotinic acid at 200 μmol per kg body weight (150 μl). Triglyceride levels were determined by a colorimetric method (Sigma). All animal experiments and care were approved by the local Animal Care & Use Committee.

Note: Supplementary information is available on the Nature Medicine *website.*

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Competing interest statement

The authors declare that they have no competing financial interests.

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Supplementary Methods

Determination of cAMP levels and ERK activity

Intracellular cAMP levels were determined under the indicated conditions with a radioreceptor assay using [^{3}H]-cAMP (Amersham) in CHO-K1 cells transiently cotransfected with the β_2-adrenergic receptor and PUMA-G or HM74 in 6-well plates.

Erk activity was determined by measuring the phosphorylation of Erk1/2. After starvation for 12 h, CHO-K1 cells transiently transfected with PUMA-G or HM74 receptors were incubated under the indicated conditions for 5 minutes at 37°C. Cells were lysed [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 % (v/v) NP-40, 0,5 % (w/v) sodium desoxycholate, 0.1 % (w/v) SDS and protease inhibitors], and samples were analyzed by immunoblotting using anti-phospho-ERK1/2 antibodies (Cell Signalling) and an electrochemiluminescence (ECL) detection system (Roche).

Construction of PUMA-G targeting vector

The targeting vector was constructed as shown in Fig. 3a. The open reading frame was replaced by a β-galactosidase expression cassette and the HSV-tk-promotor-driven neomycin gene resistance cassette. For negative selection, a viral-thymidine-kinase cassette was inserted into the targeting vector. Gene targeting in E14.1 ES cells was performed as described[1]. Homologous recombinant ES cell clones were detected by PCR using the primers PUMA-G-sense-1 (5´-TCAGATCTGACTCGTCCACC-3´) and 333-KO (5´-CCTCTTCGCTATTACGCCAGC–3´) and were confirmed by Southern Blot analysis (SpeI digest) using the 5´flanking probe. Single integration of the targeting vector was verified by Southern Blot using a neomycin probe (BamHI digest). Two independently derived ES cell clones were injected into C57BL/6 blastocysts. Germline transmission was confirmed by PCR and Southern blot analysis.

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Molecular Identification of High and Low Affinity Receptors for Nicotinic Acid*

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Alan Wise,[a,b] Steven M. Foord,[c] Neil J. Fraser,[a,d] Ashley A. Barnes,[e] Nabil Elshourbagy,[f] Michelle Eilert,[g] Diane M. Ignar,[g] Paul R. Murdock,[h] Klaudia Steplewski,[h] Andrew Green,[a] Andrew J. Brown,[a] Simon J. Dowell,[e] Philip G. Szekeres,[e] David G. Hassall,[i] Fiona H. Marshall,[a,j] Shelagh Wilson,[a] and Nicholas B. Pike[i]

From the Departments of [a]7TMR Systems Research, [c]Target Bioinformatics, [e]Gene Expression and Protein Biochemistry, [h]Cellular Genomics, and [i]Cardiovascular and Urinary Centre of Excellence for Drug Discovery, GlaxoSmithKline, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY, United Kingdom, the [g]Department of Metabolic Diseases, Metabolic and Viral Diseases Centre of Excellence for Drug Discovery, GlaxoSmithKline, Research Triangle Park, North Carolina 27709, and [f]Gene Cloning and Expression Proteomics, GlaxoSmithKline, King of Prussia, Pennsylvania 19406-0939

Nicotinic acid has been used clinically for over 40 years in the treatment of dyslipidemia producing a desirable normalization of a range of cardiovascular risk factors, including a marked elevation of high density lipoprotein and a reduction in mortality. The precise mechanism of action of nicotinic acid is unknown, although it is believed that activation of a G_i-G protein-coupled receptor may contribute. Utilizing available information on the tissue distribution of nicotinic acid receptors, we identified candidate orphan receptors. The selected orphan receptors were screened for responses to nicotinic acid, in an assay for activation of G_i-G proteins. Here we describe the identification of the G protein-coupled receptor HM74 as a low affinity receptor for nicotinic acid. We then describe the subsequent identification of HM74A in follow-up bioinformatics searches and demonstrate that it acts as a high affinity receptor for nicotinic acid and other compounds with related pharmacology. The discovery of HM74A as a molecular target for nicotinic acid may facilitate the discovery of superior drug molecules to treat dyslipidemia.

Nicotinic acid has been used in the treatment of dyslipidemia for many years, producing a very desirable modification of multiple cardiovascular risk factors, increasing high density lipoprotein, and decreasing very low density lipoprotein, low density lipoprotein, triglycerides, and lipoprotein (a), which results in a reduction in mortality (1). Despite its long history of clinical use, the precise mechanism of action of nicotinic acid is unknown, although it is believed that inhibition of adipocyte lipolysis via the activation of a G_i-coupled receptor may contribute (2–4). It has been postulated that a reduction in free fatty acids liberated from adipose tissue results in a reduction of hepatic triglycerides available for very low density lipoprotein and low density lipoprotein synthesis, which in part explains the hypolipidemic effects observed during nicotinic acid therapy. Because the identification of a molecular target for nicotinic acid would facilitate our understanding of its mode of action and potentially enable the discovery of superior drug molecules, we instigated a strategy to identify this receptor. To identify the G_i-G protein-coupled receptor for nicotinic acid, orphan receptors were selected based on their tissue expression profiles for a rational screening exercise. Recently, the pharmacological sites of action of nicotinic acid were shown to be largely restricted to adipose tissue and spleen (5). Therefore, to identify this nicotinic acid receptor, we selected a subset of 10 orphan G protein-coupled receptors, which by mRNA distribution analysis (TaqMan) exhibited significant expression levels in both adipose tissue and spleen. These receptors were then expressed in an appropriate mammalian cell line to allow measurement of a functional response (GTPγS[1] binding) following nicotinic acid treatment. This paper describes the identification of HM74 as a low affinity receptor for nicotinic acid and the subsequent indication of HM74A, a high affinity receptor for nicotinic acid. The identification of HM74A has allowed us to test additional compounds that have been reported to possess a similar pharmacology to nicotinic acid.

EXPERIMENTAL PROCEDURES

Materials—Nicotinic acid, nicotinuric acid, and nicotinamide were obtained from Sigma-Aldrich, 5-methyl nicotinic acid was from Maybridge, and pyridine-3-acetic acid was from ICN. LipofectAMINE, Dulbecco's modified Eagle's medium, and fetal calf serum were from Invitrogen. [^{35}S]GTPγS (1160 Ci/mmol) and [5,6-^{3}H]nicotinic acid (50–60 Ci/mmol) were purchased from Amersham Biosciences and Biotrend, respectively. Pertussis toxin was from Sigma-Aldrich. Acipimox, Acifran, and 5-methyl pyrazole-3-carboxylic acid were synthesized by chemists within GlaxoSmithKline.

Molecular Biology—The HM74 expressed sequence tag was identified from the public data base as a potential seven transmembrane-spanning receptor, and the predicted open reading frame was amplified using human placenta cDNA as template. Comparison of the nucleotide sequence of HM74 with that of the published sequence revealed 15 nucleotide differences as well as a 5-nucleotide insertion at the 3′ end of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY148884 and EMM_patAR098624.

[b] To whom correspondence should be addressed: 7TMR Systems Research, GlaxoSmithKline, Medicines Research Centre, Gunnels Wood Rd., Stevenage, Hertfordshire, SG1 2NY, UK. Tel.: 1438-764988; Fax: 1438-768091; E-mail: alan.x.wise@gsk.com.

[d] Present address: Pharmaceutical Discovery, Lexicon Genetics Inc., 8800 Technology, Forest Place, The Woodlands, Houston, TX 77381-1160.

[j] Present address: University of Cambridge, Dept. of Pharmacology, Tennis Court Rd., Cambridge CB2 1QJ, UK.

[1] The abbreviations used are: GTPγS, guanosine 5′-(γ-thio)triphosphate; GIRK, G protein-regulated potassium channel; CHO, Chinese hamster ovary.

the clone that resulted in a different 3′ coding sequence. The cloning procedure was performed twice more to confirm the changes in the amino acid sequence. To confirm the correct initiation methionine, a cDNA clone containing the entire coding region and the 5′-untranslated region was isolated using human placenta cDNA library. Sequence analysis of the clone, which we termed HM74A, showed the presence of a stop codon prior to the first initiation methionine. A murine sequence with significant homology to human HM74 was identified by searching public domain data bases with the peptide sequence for human HM74 taken from GenBank™ accession number D10923. A TBLASTN search produced significant alignment with accession numbers AJ300198 and AJ300199, which encode the *Mus musculus* PUMA-G gene for a putative seven transmembrane-spanning receptor (termed mHM74A). Using the human and murine sequence information, the PCR was used to amplify the corresponding rat gene. The accession number for human HM74A is AY148884. The cDNA sequence of rat HM74A is partially represented by EMM_patAR098624.

TaqMan mRNA Analysis—Poly(A)+ RNA from 20 tissues of four different individuals (two males, two females except prostate) was prepared, reverse transcribed, and analyzed by TaqMan quantitative PCR as described previously (6). Briefly, 1 μg of poly(A)+ RNA was reverse transcribed using random priming, and the cDNA produced was used to make up to 1,000 replicate plates with each well containing the cDNA from 50 ng of poly(A)+ RNA. TaqMan quantitative PCR (Applied Biosystems, Warrington, UK) was used to assess the level of each gene relative to genomic DNA standards. The data are presented as the means of mRNA copies detected per ng of poly(A)+ RNA from four individuals ± S.E. ($n = 4$). The gene-specific reagents were: HM74, forward primer, 5′-ACTACTATGTGCGGCGTTCAGAC-3′, and reverse primer, 5′-GGCGGTTCATGGCAAACA-3′; TaqMan probe, 5′-ACCAGCCGGCAAGGGATGTCC-3′; HM74A, forward primer, 5′-ACAACTATGTGAGGCGTTGGGA-3′, and reverse primer, 5′-TGGCGGTTCATAGCCAACA-3′; TaqMan probe, 5′-ATCAGCCGGCAAGGGATGTCC-3′; GPR81, forward primer, 5′-TCGGATGAAGAAGGCGACC-3′, and reverse primer, 5′-GCTGGGCAGGTAGCATGTG-3′; and TaqMan probe, 5′-TGAACACAATTGCCACCACCATGTG-3′.

Cell Biology—For transient transfections, HEK293T cells (HEK293 cells stably expressing the SV40 large T-antigen) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2 mM glutamine. The cells were seeded in 90-mm culture dishes and grown to 60–80% confluence (18–24 h) prior to transfection with vectors containing the relevant DNA inserts using LipofectAMINE reagent. For transfection, 9 μg of DNA was mixed with 30 μl of LipofectAMINE in 0.6 ml of Opti-MEM (Invitrogen) and was incubated at room temperature for 30 min prior to the addition of 1.6 ml of Opti-MEM. The cells were exposed to the LipofectAMINE/DNA mixture for 5 h, and 6 ml of 20% (v/v) fetal calf serum in Dulbecco's modified Eagle's medium was then added. The cells were harvested 48 h after transfection. Pertussis toxin treatment was carried out by supplementation into the medium at 50 ng ml^{-1} for 16 h. All of the transient transfection studies involved co-transfection of receptor together with the $G_{i/o}$ G protein, $G\alpha_{o1}$.

For the generation of stable cell lines, the above method was used to transfect CHO-K1 cells seeded in six-well dishes grown to 30% confluence. These cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium containing 10% fetal calf serum and 2 mM glutamine. 48 h post-transfection the medium was supplemented with 400 μg/ml G418 for selection of antibiotic resistant cells. Clonal CHO-K1 cell lines stably expressing HM74A were confirmed by [^{35}S]GTPγS binding measurements, following the addition of nicotinic acid.

P2 Membrane Preparation—Plasma membrane-containing P2 particulate fractions were prepared from cell pastes frozen at −80 °C after harvest. All of the procedures were carried out at 4 °C. The cell pellets were resuspended in 1 ml of 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5 (buffer A), and by homogenization for 20 s with a Ultra Turrax followed by passage (5 times) through a 25-gauge needle. The cell lysates were centrifuged at 1,000 × *g* for 10 min in a microcentrifuge to pellet the nuclei and unbroken cells, and P2 particulate fractions were recovered by microcentrifugation at 16,000 × *g* for 30 min. P2 particulate fractions were resuspended in buffer A and stored at −80 °C until required.

[^{3}H]Nicotinic Acid Binding—Saturation binding assays were carried out on plasma membrane-containing P2 particulate fractions from HEK293T cells transiently co-expressing HM74A and $G\alpha_{o1}$ using ^{3}H-labeled nicotinic acid as described (5). Briefly, the membranes (10 μg/point) were incubated with increasing concentrations of [5,6-^{3}H]nicotinic acid (60 Ci/mmol; Biotrend) for 3 h at room temperature with agitation. The assay was performed in 50 mM Tris-HCl pH 7.4 binding buffer containing 1 mM $MgCl_2$ in a total volume of 500 μl. Nonspecific binding was assessed in the presence of 1 mM nicotinic acid. Membrane-bound ligand was recovered onto presoaked GF/B filters using a Brandel 48-well harvester, washed four times with 1 ml of ice-cold binding buffer, and measured by liquid scintillation counting. [^{3}H]Nicotinic acid (20 nM) displacement assays were performed using plasma membrane-containing P2 particulate fractions, prepared from either a stable CHO cell line expressing recombinant human HM74A or human adipocytes (Zen-Bio) as described (5) and above.

[^{35}S]GTPγS Binding—[^{35}S]GTPγS binding assays were performed at room temperature in 96-well format as described previously (7). Briefly, the membranes (10 μg/point) were diluted to 0.083 mg/ml in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) supplemented with saponin (10 mg/l) and preincubated with 10 μM GDP. Various concentrations of nicotinic acid or related molecules were added, followed by [^{35}S]GTPγS (1170 Ci/mmol; Amersham Biosciences) at 0.3 nM (total volume of 100 μl), and binding was allowed to proceed at room temperature for 30 min. Nonspecific binding was determined by the inclusion of 0.6 mM GTP. Wheat germ agglutinin SPA beads (Amersham Biosciences) (0.5 mg) in 25 μl of assay buffer were added, and the whole was incubated at room temperature for 30 min with agitation. The plates were centrifuged at 1500 × *g* for 5 min, and bound [^{35}S]GTPγS was determined by scintillation counting on a Wallac 1450 Microbeta Trilux scintillation counter.

Oocyte Methods—Capped cRNA (20–50 ng/oocyte) was injected into stage V–VI defolliculated oocytes (8), and two microelectrode voltage clamp recordings were made 3–7 days post-RNA injection from a holding potential of −60 mV. The oocytes were superfused with ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5 at 25 °C) at a flow rate of 2 ml min^{-1}. To facilitate the recording of GIRK1/GIRK4 potassium currents, the extracellular solution was switched to a high potassium solution (90 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES). The recording electrodes had a resistance of 0.5–1.0 MΩ when filled with 3 M KCl. The measurements of potassium currents were made from two batches of oocytes harvested on different days from different toads. Nicotinic acid was applied by addition to the superfusate, and cumulative concentration response curves were constructed for each individual oocyte tested.

Yeast—Human HM74A was subcloned into p426GPD adjacent to the promoter (9), transferred to pRS306, and integrated into the *ura3* locus of MMY16 (10). β-Galactosidase assays to measure *FUS1-lacZ* reporter gene induction were performed as described (11) except that nicotinic acid was omitted from the assay mix, and the substrate fluorescein-β-D-galactopyranoside (Molecular Probes; final concentration, 20 μM) was used in place of chlorophenol red-β-D-galactosidase.

RESULTS

Nicotinic acid-mediated stimulation of [^{35}S]GTPγS binding was observed only in membranes from HEK293T cells co-transfected with the cDNA for HM74 and the G protein $G\alpha_{o1}$ (Fig. 1*A*). The nicotinic acid-induced stimulation was concentration-dependent and was found to be abolished following pretreatment of cells with pertussis toxin (50 ng ml^{-1} for 16 h), suggesting that the effect was $G_{i/o}$ G protein-mediated (Fig. 1*B*). However, the half-maximal effector concentration for nicotinic acid was estimated to be in excess of 1 mM, over 1000-fold higher than that previously reported in rat adipose tissue and spleen membranes (5). Subsequent to the identification of HM74 as a low affinity receptor for nicotinic acid, we utilized a molecular biology approach to identify a novel paralogue of HM74, termed HM74A. Comparison of the nucleotide sequences of HM74A and HM74 revealed 15 base changes as well as a 5-nucleotide insertion at the 3′ end of the clone resulting in HM74A possessing a shortened C-terminal tail (Fig. 2*A*). The two receptors are highly homologous, displaying 96% identity at the protein level and differing by only 15 amino acids. A third gene, GPR81, previously identified by customized searching of the GenBank™ high throughput genomic sequences data base (12), was also found to exhibit substantial homology to HM74 and HM74A (57 and 58% amino acid sequence identity, respectively). Despite their high degree of similarity, HM74 and HM74A are not simply polymorphic variants but are separate genes being co-located with GPR81 at chromosome

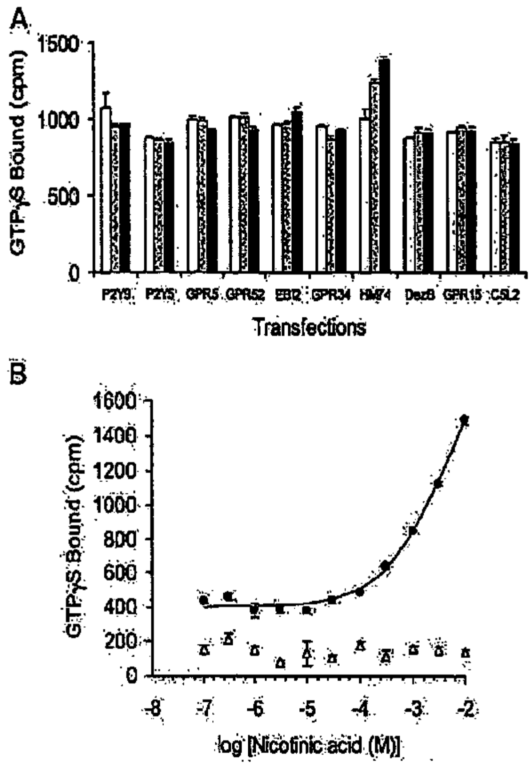

FIG. 1. HM74 is a G protein-coupled receptor that responds to nicotinic acid. *A*, application of 300 μM (*hatched columns*) and 1 mM (*filled columns*) nicotinic acid to membranes from HEK293T cells expressing a variety of orphan G protein-coupled receptors was found to stimulate [^{35}S]GTPγS binding in membranes from cells expressing HM74 only (*open columns*, basal conditions). *B*, nicotinic acid stimulated a dose-dependent increase in [^{35}S]GTPγS binding in cells expressing HM74 (*filled circles*) that was ablated by pretreatment with 50 ng ml^{-1} pertussis toxin for 16 h prior to harvest (*triangles*). All of the transient transfection studies involved co-transfection of receptor together with the $G_{i/o}$ G protein, $G\alpha_{o1}$.

12q24.31 (part of this region of chromosome 12 is represented by accession number AC026362). Messenger RNA expression profiling of HM74 and HM74A using TaqMan quantitative reverse transcriptase-PCR analysis with probes designed and confirmed to discriminate between these two homologous receptors (data not shown) showed that both exhibited similar distribution patterns that were largely restricted to adipose tissue and spleen (Fig. 2*B*). Hence, the expression patterns of HM74A and HM74 are concomitant with that of a nicotinic acid receptor. Interestingly, GPR81 appears to be highly restricted to adipose tissue.

Expression of HM74A together with $G\alpha_{o1}$ in HEK293T cells gave robust concentration-dependent responses to nicotinic acid with a half-maximal concentration (EC_{50} = 250 ± 27 nM) similar to that observed in rat adipose tissue and spleen membranes (5) (Fig. 3*A*). Conversely, GPR81 responded to nicotinic acid only at relatively high concentrations (10 mM). Expression of HM74A, but not HM74 or GPR81, also produced saturable specific binding of ^{3}H-labeled nicotinic acid with an affinity (K_d 95.8 ± 9.5 nM) similar to that recorded from rat adipose tissue and spleen membranes (6) (Fig. 3*B*). We have not been able to quantify the levels of expression of HM74 and GPR81 because of their low affinity for nicotinic acid. Next, we expressed HM74A and HM74 in *Xenopus* oocytes to study their coupling to the G_i-G protein-regulated potassium channels GIRK1 and GIRK4 (13). Concentration-dependent responses to nicotinic acid with a half-maximal concentration (EC_{50}) of 130 ± 50 nM were observed for HM74A (four oocytes). Responses to nicotinic

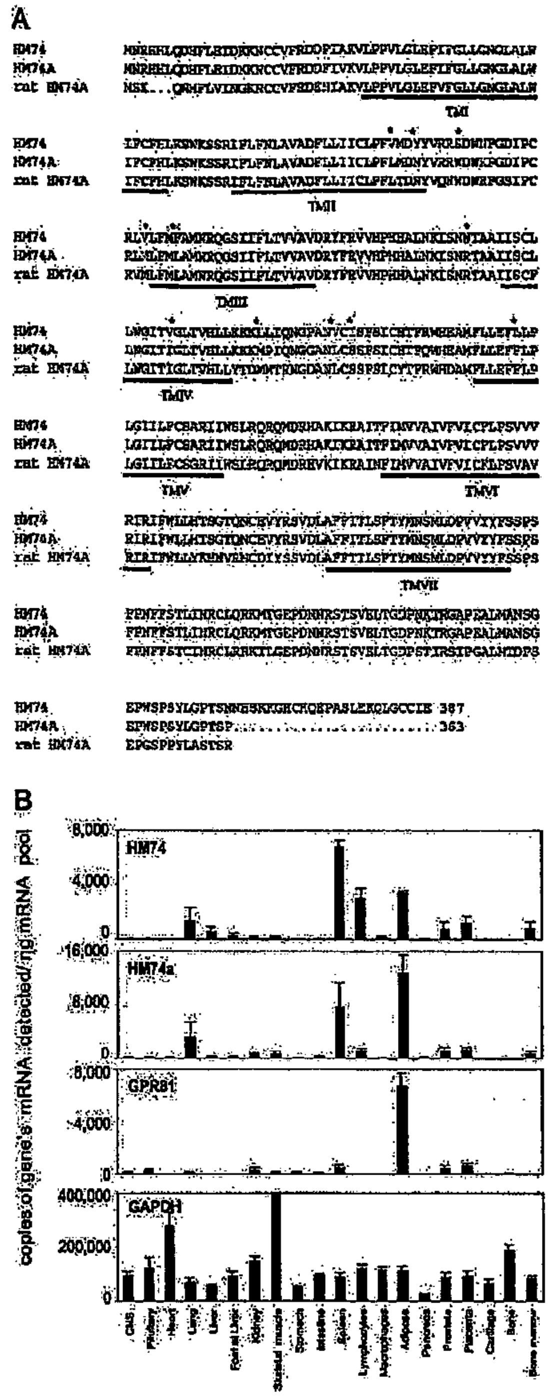

FIG. 2. HM74 and HM74A are highly homologous proteins with similar mRNA tissue distribution patterns and chromosomal location. *A*, amino acid sequences of human HM74 and human and rat HM74A aligned for comparison. Residues sharing identity between human and rat HM74A but not with HM74 are indicated with *asterisks*. *B*, TaqMan quantitative reverse transcriptase-PCR analysis of mRNA levels in human tissues. The cDNA from the reverse transcription of 1 ng of poly (A)$^+$ RNA from multiple tissues for four different nondiseased individuals was assessed for its HM74, HM74A, and GPR81 and housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), mRNA levels. The data are presented as the means (± S.E.) mRNA levels from four individuals for each tissue.

acid were also obtained at concentrations of 100 μM and above following expression of HM74 (5 oocytes) (Fig. 3*C*). Finally, we investigated coupling to the pheromone response pathway of

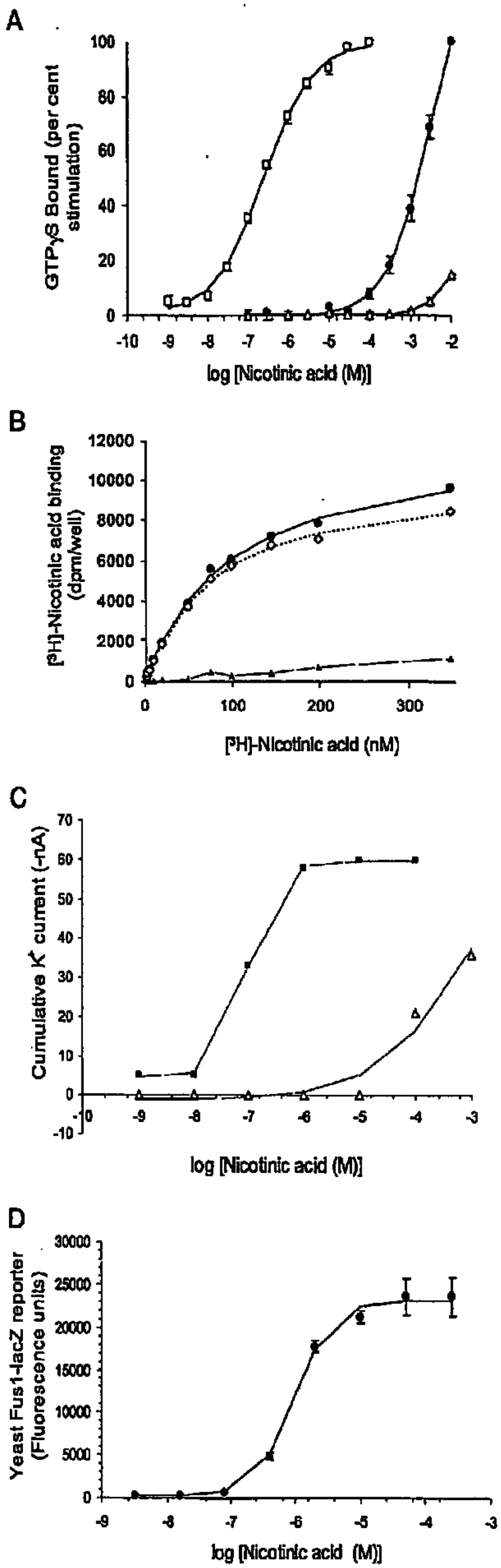

FIG. 3. Radioligand binding studies and functional studies with nicotinic acid. *A*, stimulation by nicotinic acid of [^{35}S]GTPγS binding in HM74-expressing (*filled circles*), HM74A-expressing (*squares*), and GPR81-expressing (*triangles*) membranes. The data are normalized to the peak response to nicotinic acid at HM74A. All of the transient transfection studies in HEK293T cells involved co-transfection of receptor together with Gα_{o1}. *B*, radioligand binding analysis with ^{3}H-labeled nicotinic acid by saturation isotherm of human HM74A. *Filled circles*, total binding; *filled triangles*, nonspecific binding; *diamonds*, specific binding. The results shown exemplify n = 3, with each experiment performed in duplicate. *C*, oocyte data. Representative cumulative concentration response curves to nicotinic acid are shown for different individual oocytes expressing either HM74 (*triangles*) or HM74A (*filled squares*) in combination with the potassium channels GIRKs 1 and 4. *D*, activation of the yeast pheromone response pathway by nicotinic acid in yeast expressing recombinant human HM74A. The results show the means of two independent yeast isolates, each determined with n = 6.

TABLE I

Comparison of functional activity of nicotinic acid analogues at human and rat HM74A

Potencies are given as means of the EC$_{50}$ obtained from three separate experiments (± S.E.).

Compound	EC$_{50}$	
	Human HM74A	Rat HM74A
	μM	
Nicotinic acid	0.25 ± 0.027	0.4 ± 0.05
3-Pyridine-acetic acid	5.5 ± 0.95	13.7 ± 3.2
5-Methylnicotinic acid	8.7 ± 4.0	22.4 ± 5.2
Nicotinamide	>1000	>1000
Nicotinuric acid	>1000	>1000
3-Cyanopyridine	inactive	inactive

Saccharomyces cerevisiae, measuring receptor activation with a reporter gene. Using nicotinic acid-free growth medium, we demonstrated concentration-dependent activation of HM74A in response to nicotinic acid (EC$_{50}$ = 904 ± 28 nM; Fig. 3*D*). The optimal agonist responses were observed with chimeric yeast/mammalian Gα subunits having the C-terminal 5 amino acids of G$_i$ or the promiscuous Gα, G$_{16}$ (Fig. 3*D* and data not shown) (11). The yeast data confirm that HM74A is sufficient to confer the nicotinic acid response, because these cells lack endogenous G protein-coupled receptors capable of activating this pathway.

A number of nicotinic acid analogues were employed to characterize HM74A using the [^{35}S]GTPγS binding assay (Table I). Similar rank orders of potency were found at HM74A compared with those previously described in native rat tissue (5), whereas all of the analogues displayed either no or very weak activity at HM74 (data not shown). All of the analogues were also inactive at GPR81 (data not shown). Furthermore, we also cloned the rat orthologue of HM74A, which was found to exhibit 82% identity at the protein level with its human counterpart (Fig. 2*A*). As expected, no significant pharmacological differences were observed between recombinantly expressed rat and human HM74A (Table I).

Acipimox (Olbetam) and Acifran (AY-25,712) are two molecules that have been reported to produce a pharmacological profile resembling that of nicotinic acid in rat and human studies (14–17) (Fig. 4*A*). Using the [^{35}S]GTPγS binding assay, we found that Acipimox was a full agonist at HM74A (EC$_{50}$ = 6 ± 1 μM) and exhibited weak activity at HM74 and no activity at GPR81. Acifran acted as a full and relatively potent agonist at both HM74A (EC$_{50}$ = 2.1 ± 0.2 μM) and HM74 (EC$_{50}$ = 20 ± 4 μM) but showed no significant agonism at GPR81 up to 1 mM (Fig. 4*B*). Acifran also activated HM74A in yeast (EC$_{50}$ = 3.0 ± 0.08 μM). The fact that Acifran has been identified as a high affinity ligand for HM74 (Fig. 4*B*) strongly suggests that our transfection system is sufficiently efficient to allow agonist profiling. Furthermore, the signal to noise ratios observed with Acifran at both HM74A and HM74 are similar, which suggests

that these receptors are expressed at similar levels. In addition, we have expressed HM74A and HM74 in a range of different systems (mammalian, yeast, and oocyte), and in all of these expression systems there is an ~1000-fold separation in the potency of nicotinic acid, suggesting that this is a real observation.

Acifran and Acipimox were included in a group of molecules with structural or pharmacological similarities with nicotinic acid that were tested in a [^{3}H]nicotinic acid displacement assay performed in membranes from either a stable CHO cell line expressing recombinant human HM74A or human adipocytes (Table II). The rank order of potency for the displacement of [^{3}H]nicotinic acid binding was nicotinic acid > 5-methyl pyrazole-3-carboxylic acid = pyridine-3-acetic acid > Acifran > 5-methyl nicotinic acid = Acipimox ≫ nicotinuric acid = nicotinamide. This rank order of potency was the same in both the stable CHO cell line expressing recombinant human HM74A and human adipocytes.

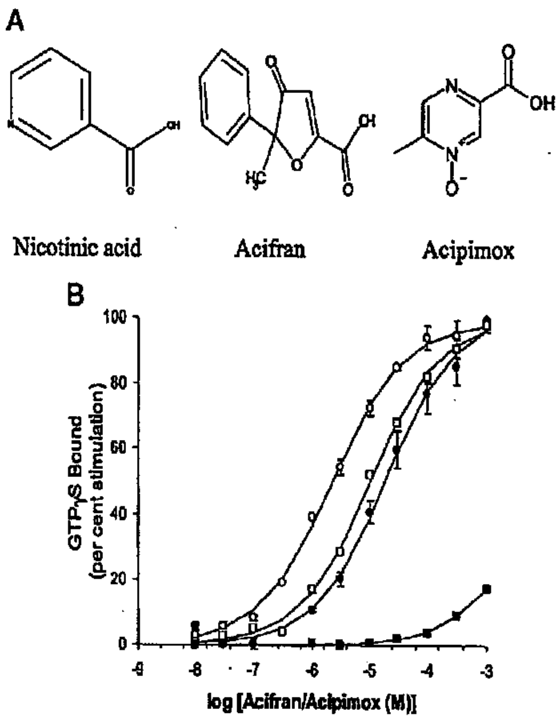

FIG. 4. **Acifran and Acipimox activity at HM74 and HM74A.** *A*, a comparison of the structures of nicotinic acid, Acipimox and Acifran. *B*, stimulation by Acifran (*circles*) and Acipimox (*squares*) of [^{35}S]GTPγS binding in HM74-expressing (*filled circles* and *filled squares*) and HM74A-expressing (*open circles* and *open squares*) membranes. The data are normalized to the peak responses to Acifran and Acipimox at HM74A. All of the transient transfection studies involved co-transfection of receptor together with the $G_{i/o}$ G protein, $G\alpha_{o1}$.

DISCUSSION

HM74 was identified as a low affinity receptor for nicotinic acid following the screening of a panel of orphan receptors selected because of their tissue expression profile. HM74 is an orphan receptor that had been previously cloned from a cDNA library derived from human monocytes (18). The half-maximal effector concentration for nicotinic acid at HM74 was estimated to be in excess of 1 mM, ~1000-fold higher than that previously reported in membranes produced from rat adipose tissue or spleen (5). We considered three possible explanations for this discrepancy in nicotinic acid potency. First, close homologues of HM74 may act as higher affinity nicotinic acid receptors. Second, because G protein-mediated nicotinic acid effects on native tissue have almost always been recorded from rat, variation between human and rodent receptors may explain this phenomenon. Finally, differences in the pharmacological integrity of the recombinantly expressed receptor and its endogenously expressed counterpart may explain potency changes.

A molecular biology approach resulted in the identification of a novel paralogue of HM74, termed HM74A. Despite their high degree of similarity, HM74 and HM74A are not simply polymorphic variants but are separate genes being co-located with GPR81 at chromosome 12q24.31. TaqMan analysis confirmed that the expression pattern of HM74A was very similar to HM74. When expressed in a variety of test systems, HM74A was confirmed as a high affinity receptor for nicotinic acid. The activity and affinity of nicotinic acid was in good agreement with that previously reported in the literature (5). Furthermore, following the cloning of the rat orthologue of HM74A, we found no significant pharmacological differences between nicotinic acid derivatives tested against either human or rat HM74A. The murine variant of HM74A, PUMA-G, was recently reported to be an interferon γ-inducible gene in macrophages, suggesting a possible role in macrophage function (19). This finding is further supported by a recent report describing a nicotinic acid receptor in a murine macrophage cell line (20). Based on the TaqMan data generated for the distribution of human HM74A, there appears to be little or no expression in macrophages (Fig. 2*B*). This may indicate that species differences in the distribution of HM74A exist or is a reflection of the activation state of the macrophages used in this experiment. It will be of interest to determine whether the expression of HM74A can be up-regulated in human macrophages following incubation with interferon γ.

In the [^{3}H]nicotinic acid displacement assay, both the absolute potency and the rank order of potency of the HM74A ligands studied was the same, whether tested against the stable CHO cell line expressing recombinant human HM74A or human adipocytes. These data strongly suggest that HM74A is the G_i-G protein-coupled nicotinic acid receptor on human adipocytes. Acipimox and Acifran have also been identified as full

TABLE II

A comparison of the potency of a range of HM74A agonists at displacing [^{3}H]nicotinic acid from either CHO membranes expressing recombinant human HM74A or human adipocyte membranes

	Mean IC_{50} ± S.E. for displacement of [^{3}H]nicotinic acid from CHO membranes expressing human HM74A	Mean IC_{50} ± S.E. for displacement of [^{3}H]nicotinic acid from human adipocyte membranes
	μM	μM
Nicotinic acid	0.081 ± 0.003 (n = 14)	0.079 ± 0.003 (n = 8)
5-Methyl pyrazole-3-carboxylic acid	0.525 ± 0.041 (n = 11)	0.518 ± 0.022 (n = 4)
Pyridine-3-acetic acid	0.536 ± 0.052 (n = 6)	0.563 ± 0.061 (n = 4)
Acifran	1.12 ± 0.052 (n = 8)	0.833 ± 0.050 (n = 4)
5-Methyl nicotinic acid	4.12 ± 0.063 (n = 7)	3.68 ± 0.66 (n = 3)
Acipimox	5.10 ± 0.039 (n = 6)	4.34 ± 0.51 (n = 4)
Nicotinuric acid	70.5 ± 2.76 (n = 7)	63.8 ± 6.20 (n = 3)
Nicotinamide	92.3 ± 4.72 (n = 7)	75.3 ± 3.41 (n = 3)

agonists at HM74A. These compounds have also been reported to produce a pharmacological effect resembling that of nicotinic acid in rat and human studies (14–17). The other compounds identified that displace nicotinic acid from HM74A, 5-methyl pyrazole-3-carboxylic acid, pyridine-3-carboxylic acid, and 5-methyl nicotinic acid, have all previously been shown to inhibit adipocyte lipolysis (2, 23). Nicotinamide, which unlike nicotinic acid produces no alteration in lipoprotein profiles (22), acted only as a very weak agonist at HM74A. Indeed, nicotinamide was ~1000-fold less potent than nicotinic acid, a level of activity that could be due to contaminant nicotinic acid (*e.g.* 0.1%). It would appear that activation of HM74A would account for the inhibition of lipolysis observed with these compounds. Therefore, of the compounds that have been tested in man, it would appear that potency at HM74A is linked with their efficacy at normalizing lipoprotein profiles.

We have demonstrated that HM74A is a high affinity receptor for nicotinic acid and believe that this receptor is a likely candidate as a molecule target for the beneficial therapeutic effects observed with nicotinic acid. Nicotinic acid is an effective therapeutic agent; however, it has to be administered at high doses and has a characteristic side effect profile defined by intense, but transient, prostaglandin-mediated cutaneous vasodilation ("flushing") that affects patient compliance (21, 22).

Unlike HM74A, we were unable to identify rodent orthologues of HM74 using conventional gene cloning strategies and bioinformatics searches. This suggests that in humans HM74 may be the result of a relatively recent gene duplication event. Furthermore, of the compounds tested, only Acifran exhibited activity at HM74. In fact, Acifran is the first molecule that we have identified to date that exhibits significant potency at HM74. Because of the high degree of homology between HM74A and HM74 and the existence of highly selective ligands, site-directed mutagenesis may be a useful strategy in determining which amino acid residues play a key role in ligand binding. Indeed, 11 amino acid residues are conserved in human and rat HM74A but not in HM74 (Fig. 2*A*). Such residues may play key roles in determining the differences in ligand binding affinities between HM74A and HM74. The identification of HM74A as a molecular target for nicotinic acid will facilitate the discovery of potent and selective ligands for this receptor and may expedite the discovery of improved anti-hyperlipidemic drug molecules.

Acknowledgments—We thank members of Discovery and Genetics Research and the Cardiovascular and Urinary Centre of Excellence for Drug Discovery for support. We also thank Dr. R. Ravid (Netherlands Brain Bank, Amsterdam, The Netherlands) for the arrangement/donation of brain tissue, Jean-Philippe Walhin for expert technical assistance, and E. Koppe for the provision of the human HM74A stable cell line.

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BIOTECHNOLOGY, CHEMICAL, & PHARMACEUTICAL
CUSTOMER PARTNERSHIP MEETING
U.S. Patent & Trademark Office
March 12, 2008

Agenda

1. Worksharing Initiatives & Accelerated Examination

    Pinchus Laufer, Legal Advisor, OPLA
    Pinchus.Laufer@uspto.gov, 571-272-7754

2. IP Protection of Plants

    Anne Marie Grünberg, SPE, Art Unit – 1661 & 1638
    Anne.Grunberg@uspto.gov, 571-272-0975

3. Patent Applications: Biotechnology & Mechanical

    Thomas Barrett, QAS TC 3700
    Thomas.Barrett@uspto.gov, 571-272-7754.

4. Enablement for Derivatives of Compositions of Matter

    James Wilson, SPE TC 1624
    James.Wilson@uspto.gov, 571-272-7754

5. Enablement in Claims to Therapeutic Treatment

    Gene.Witz, QTAS 1600
    Gene.Witz@uspto.gov @uspto.gov, 571-272-7754

6. Rejoinder Practice

    Julie Burke, QAS TC 1600
    Julie.Burke@uspto.gov, 571-272-0512

7. FY07 Restriction Petition Survey & Restriction Training Update

    Julie Burke, QAS TC 1600

Notes prepared by John N. Calve, Esq., Biotechnology Subcommittee Chair, USPTO liaison. If you have any questions or suggestions (e.g. topics for future meetings), please contact me at: jcalve_patents@verizon.net or (202)-483-6482.

I. Worksharing Initiatives & Accelerated Examination

Worksharing Initiatives

- Patent Prosecution Highway (PPH) Program
- New Route
- Strategic Handling of Applications for Rapid Examination (SHARE)
- TriWay
- PCT Partnerships

Accelerated Examination Program

1. Patent Prosecution Highway (PPH)

The goal of the PPH is to allow an applicant to "fast-track" prosecution in the second office, when the first office allows one or more claims. The second office gets the search and examination results from the first Office before conducting its examination. Applicant benefits by getting the examination results faster.

If the USPTO is the OFF and the U.S. application contains claims that are determined to be allowable, applicants may request to have the corresponding application filed in the OSF or advanced out of turn for examination in the OSF.

If the JPO, UKIPO, CIPO, or KIPO is the OFF and the application contains claims that are determined to be allowable, applicant may petition to make the U.S. application special under the PPH (pilot) program.

2. New Route

The New Route Pilot Project is a pilot program between the United States Patent and Trademark Office and the Japan Patent Office (signed 24 January 2008). The new route is a work-sharing proposal. By filing an application in one member office-deemed a filing in all member offices. All designated second offices get the search and examination results from first Office prior to "national stage."

Two filing scenarios for eligibility in program.

1. A priority application that is filed in the first office and a PCT application claiming priority to that application is filed with the same first office as the PCT receiving Office (RO).

2. There is no priority application. A PCT application that is filed with the PCT RO of the first office

When the USPTO is the office of first filing an applicant may participate in the pilot by filing a request in the JPO. www.jpo.go.jp/torikumi_e/japan_usa_newroute_e.htm The applicant must also notify the USPTO.

The other scenario where the JPO is the office of first filing (U.S. Application is a national stage entry of a PCT application filed with the JPA as the PCT receiving office. The PCT must either contain a priority claim to a single priority application filed in the JPO or contain no priority data.

For more details please refer to the link of a document on the USPTO website. The document is s pre-OG notice and can be obtained by doing a search on the USPTO homepage - "new route pilot."

http://www.uspto.gov/web/offices/pac/dapp/opla/preognotice/new_route_pilot_012008.pdf

3. Strategic Handling of Applications for Rapid Examination (SHARE)

SHARE is a proposal to implement a policy of prioritizing search and examination of first filings, with the stated goal of leveraging the work of the Office of First Filing (OFF) to enhance the throughput and quality at the Office of Second Filing (OSF).

The *SHARE* proposal would prioritize examination of applications by giving precedence in examination of applications filed with the OFF.

SHARE has a goal of leveraging work sharing to the maximum extent possible consistent with appropriate examination under each Office's statutory framework.

4. TriWay

USPTO initiative to leverage the expertise of each Office in searching its own documentation and/or documentation in its native language (e.g., Japanese documentation searched by JPO)

Each Office searches corresponding application and provides results to other offices for use in examination

5. PCT Partnerships

Outsourcing of PCT applications (Chap. I).

Designating alternative search authority for US applicants filing in Receiving Office /US or RO/IB - EPO and Korea.

6. Accelerated Examination Program

GOAL: Achieve a final decision by the examiner within 12 months from the filing date, effective August 25, 2006. Petitions prior to this date are excluded.

The new requirements apply to *all* petitions to make special, except for: petitions to make special for (i) Age and health or (ii) Patent Prosecution Highway.

The application must be: filed electronically, be complete at filing, contain 3/20 total claims or fewer directed to a single invention, include a petition, and fee unless the claims are directed to environmental quality, energy, or countering terrorism.

Please refer to the power-point presentation for more information.

2. IP Protection of Plants in the US

Statutes:

1. Plant Patent Act (1930)
   35 U.S.C. §§ 161-164

2. Plant Variety Protection Act
   7 U.S.C. §§ 2321 et seq.

3. Utility Patent to a Plant
   35 U.S.C. §§ 111

Plant Patent Act (PPA)
35 U.S.C. 161:
"Whoever invents or discovers and *asexually* reproduces any distinct and new variety of plant, including cultivated sports, mutants, hybrids, and newly found seedlings, *other than* a tuber propagated plant or a plant found in an uncultivated state, may obtain a patent ..."

History

The PPA overcame the two obstacles to patentability: "product of nature" doctrine by clarifying that "a plant discovery resulting from cultivation is unique, isolated, and is not repeated by nature..." The second obstacle was enablement, because at the time of enactment a written description could not enable a person to make a plant.

The PPA relaxed the enablement requirement by specifying that a plant patent shall not be declared invalid for failing to comply with 35 USC 112 if the description

is as *complete as is reasonably possible.* The scope of protection is for a single plant and asexual progeny.

Requirements for Patentability

1. The plant is new and distinguishable from other known varieties.

Lack of Novelty: Description of the plant in a printed publication, combined with public availability (anywhere) more than 1 year prior to filing for U.S. patent (*In re Elsner*, 381 F.3d 1125, 72 USPQ2d 1038 (Fed. Cir. 2004).

2. Plant description is as complete as is reasonably possible

Claim:

A Petunia plant substantially as described and illustrated in the specification herein.

Plant Variety Protection Act (PVPA)

- Enacted in 1970, and amended in 1994.
- Plant must be new, distinct, uniform and stable.
- In the U.S. the Act applies *only* to sexually reproduced plants and tuber propagated plants.
- 20-25 year protection from the date of grant.
- Breeder's exemption, farmer's exemption.

Requirements for PVP:

- Novelty - The plant has not been sold or otherwise disposed of for purposes of exploitation for more than one year in the United States, or more than four years in any foreign jurisdiction (six years for trees and vines).
- Distinct - distinguishable from publicly known varieties - morphological, physiological, or other characteristics (e.g., commercially valuable characteristics).

Utility Patent

- Possible to protect varieties having specific traits, plant parts, and methods of producing or using plant varieties.
- Diamond v. Chakrabarty, 447 U.S. 303 (1980).
- Ex Parte Hibberd, 227 USPQ 443 (PTO Bd. Pat. App. & Int. 1985).
- Ruled that seeds, plant tissue cultures, and the plant itself are patentable subject matter under the utility patent statute.
- J.E.M. Ag Supply, Inc. v. Pioneer Hi-Bred International, Inc., 534 U.S. 124, 60 USPQ2d 1865 (2001), (Supreme Court held newly developed plant breeds fall within the scope of §101, and utility patents are not precluded by the parallel PPA and PVPA Acts).

Claims

- Plants, Plant organs or tissue, Pollen, Ovules, Tissue or cell culture, Seeds.

Plant Utility Patent (Examples)
1. Isolated plant polynucleotides and polypeptides.
2. Isolated plant regulatory elements (e.g. promoter, transcriptional elements).
3. Expression cassettes or vectors.
4. Transgenic plants having a novel phenotype.
5. Products produced from transgenic plants.

Method Claims
1. Methods of breeding novel/nonobvious plants using traditional methods.
2. Methods of molecular plant breeding.
3. Methods of producing a transgenic plant having a novel phenotype.
4. Novel plant transformation methods.
5. Methods of plant cell and tissue culture.

Plant Utility Patent Representative Claims
Claim 1. Seed of plant variety NN deposited as ATCC Accession No. _____.
Claim 2. A plant grown from the seed of Claim 1.
Claim 3. An isolated DNA encoding protein X.
Claim 4. A method of making a transgenic plant having phenotype Y comprising transforming a plant with said DNA of Claim 3.
Claim 5. A transgenic plant produced by the method of Claim 4.

Utility (35 USC § 101)
A patent application must set forth a utility that is:
- Specific, Substantial (Real-World), and Credible (Reliability of the statement based on the logic and facts that are offered by the applicant to support the assertion of utility).

Anticipation/Novelty
Does the prior art teach:
- A plant variety with the same characteristics?
- an isolated DNA as claimed?
- a method of making a transgenic plant comprising the isolated DNA as claimed? Dependent on the breadth of the claims

Non-Obviousness
- Are the characteristics of the claimed plant variety obvious over a prior art variety when grown under different conditions?
- Are the characteristics obvious morphological variants?
- Is the claimed DNA suggested by the prior art?
- If so, is there a reason to produce a transgenic plant comprising the DNA?
- Is there an expectation of success in obtaining a transgenic plant with phenotype Y?

Evidence of Possession & Reduction to Practice

- Actual reduction to practice is not always required.
- Deposit of biological materials is not a substitute for written description.

Genus/Species

- Are the species that are described representative of the claimed genus?
- Does the applicant describe structural features that are unique to the claimed genus?
- Applicant may need to include <u>structural</u> as well as functional claim language.
- Is the phenotype of the transgenic plant described?

- *Enablement (35 USC § 112- 1st)*

Has the applicant taught how to use the plant variety, *i.e.* its agronomically useful phenotypic characteristics?

- Has the applicant taught how to use the claimed DNA?
- Has Applicant taught isolated DNAs?
    - How many DNAs has the applicant isolated?
    - Has the applicant provided <u>specific</u> guidance for isolation of other functionally related DNAs, including structurally unrelated DNAs?
- Applicant may need to include <u>structural</u> as well as functional claim language.
- If the DNA is not enabled throughout the scope of the claim, the method of making a transgenic plant is not enabled throughout the scope of the claim.
- Has Applicant provided guidance for making a transgenic plant having phenotype Y?
- Have related genes resulted in phenotype Y upon expression in plants?
- An invention may support both a utility patent and a plant patent, so long as the subject matter protected by the two patents is not identical.
- Utility Patent- may be useful when the invention is not limited to a particular variety or to obtain method claims.
- Plant Patent- may be useful where it is difficult to meet the written description or enablement requirements of a utility patent.

MPEP1613 Right of Priority Based upon Application for Plant Breeder's Rights
According to **<u>35 U.S.C. 119(f)</u>**, an application for a patent may rely upon an application for plant breeder's rights filed in a WTO member country (or in a foreign UPOV Contracting Party) for priority under **<u>35 U.S.C. 119(a)</u>** through (c).

∗http://www.uspto.gov/web/offices/pac/plant/index.html
∗http://www.uspto.gov/web/offices/pac/utility/utility.htm
∗http://www.ams.usda.gov/Science/PVPO/PVPindex.htm
∗571-272-1600 – Technology center 1600 directory

3. Patent Applications: Biotechnology & Mechanical

Intersection of Biotechnology and Mechanical Arts

Class 435 is a biotechnology class associated with the mechanical arts. Class contains over 172,000 patents & published applications in class 435;
Over 8,000 disclose at least one of the following delivery devices: stent, prosthetic, or prosthesis.

o Class 623 is a mechanical class associated with biotechnology arts. Prosthesis parts, aids and accessories.

o About 30,000 patents & published applications, including stents, prosthetics and prostheses.

o Stents and prosthetics are devices found in the mechanical art that can be used for delivery of pharmaceutical or medicinal compositions.
o Example: Stents are coated with therapeutic compositions such as anti-thrombotics, antibiotics, and anti-inflammatories.

Obviousness
o If a therapeutic composition is disclosed in the prior art (e.g., anti-thrombotics or anti-inflammatories) *and* the stent is not novel the combination *may* be obvious.
Claim:
A vascular stent graft comprising:
o a biologically active surface which exhibits cell attachment activity and growth activity, the surface having linked thereto the expressed protein of a vector containing a DNA sequence of cDNA coding for the A chain of laminin.

Classification
I. Controlling Claim
If the claimed inventions classified separately, the ***controlling claim***:
a) Determines the class for the original classification and,
b) Determines the class where a patent application is to be assigned for examination

The controlling claim is determined by the principles arranged in order of precedence.
(1) The Most Comprehensive Claim
(2) Hierarchy of Categories of Subject Matter
(3) Superiority of types of subject matter
(4) Class Superiority

Most Comprehensive Claim

A claim to a combination will take priority over a subcombination claim or a claim that merely narrows an element of the combination.

Example:
1. A coating for a vascular graft having a polymeric external surface comprising: a biologically active surface which exhibits cell attachment activity and growth activity, said surface having linked thereto the expressed protein of a vector.
2. The coating of claim 1, wherein said vector contains a DNA sequence of cDNA coding for the A chain of laminin.
3. The coating of claim 1, in combination with a vascular graft.

Claim 1 is the most comprehensive claim and would be classified and sent to the appropriate art unit. A Supervisor in the art unit can transfer the application to another art unit that could "*best examine*" the application.

Reasons Supporting a Transfer of an Application
An application containing a hybrid claim a product is defined merely in terms of the process for producing it (product by process).
Where an application properly assigned to a mechanical class contains at least one claim to mixed subject matter, a part of which is biotechnical, the application *may* be assigned to the appropriate biotechnology art unit for examination.

PCT Applications
If a U.S. national application was examined, then the PCT application that claims priority to the national application will typically be assigned to the same examiner.

Otherwise if the U.S. national application and a corresponding PCT application are co-pending then both applications will be assigned, search and examination, to the examiner to whom the PCT application would normally be assigned on the basis of the first claimed invention, or to the examiner's art unit in his/her absence.

Resources for Classification include MPEP 902-903.09(a), and the Examiner's Handbook to the U.S. Patent Classification System. Available online at: <u>www.uspto.gov/web/offices/pac/dapp/sir/co/examhbk/index.htm</u>.

Within the overlap of biotechnology and mechanical arts, an issue sometimes occurs wherein a claim recites an apparatus with certain elements "attached to" the human body or specific body parts.

Functional Recitations
Limitations to parts of the human body presents no problem as long as the language is recited in the format "adapted to be attached" or "for attachment to" or in some similar way which does not positively set forth the human body <u>or portions thereof</u> as part of the claimed subject matter.

Claimed Combination

In situations where the portion of the human body is actually part of the claimed combination?

MPEP 2105- Patentable Subject Matter

Animals

On April 7, 1987, the Commissioner of Patents and Trademarks issued a notice on the policy - the Office "would now consider non-naturally occurring non-human multi-cellular living organisms, including animals, to be patentable subject matter within the scope of 35 U.S.C. 101."

A "claim directed to or including within its *scope a human being* is prohibited. Thus, when a claim is drawn to an apparatus "attached to" the human body or any part of the body it will be rejected under 35 U.S.C. 101.

112 1st Paragraph - Claims that contain language "attached to" a part of the body do not inherently raise questions of enablement or indefiniteness.

Acceptable

An intravascular stent that is permanently implanted in the vessel lumen of a patient and which is used for locally delivering genes in a vessel comprising: (a) a substrate, (b) a coating adhering to the substrate, and (c) a genetic material which is adsorbed to the surface of the coating, wherein the coating comprises a matrix of randomly interconnected protein molecules comprising one or more species of protein.

Unacceptable

An intravascular stent *for* permanent implantation in the vessel lumen of a patient and which is used for locally delivering genes in a vessel comprising: (a) a substrate, (b) a coating adhering to the substrate, and (c) a genetic material which is adsorbed to the surface of the coating, wherein the coating comprises a matrix of randomly interconnected protein molecules comprising one or more species of protein.

4. Enablement in Claims to Therapeutic Treatment

- Prodrugs 9204 Patents
- Metabolites 4340 Patents
- Polymorphs 1291 Patents
- Crystals 12,639 Patents
- Solvates 11043 Patents

Definitions

Derivatives

In chemistry, a derivative is a compound produced from an original compound either directly or by modification or partial substitution of the original compound core *or* a compound that can be imagined to arise from another compound, if one atom is replaced with another atom or group of atoms. The latter definition is common in organic chemistry. In biochemistry, the word is used about compounds that at least theoretically can be formed from the original compound.

Most frequently, compounds are set forth in the claims of patent applications along with one or more derivatized forms of the compounds claimed. Some of the most common derivatized forms of compounds seen in patent applications include:

Salts	Metabolites/Prodrugs
Isomers	Crystals/Polymorphs
Analogues	Solvates/Hydrates

Salts : Ionic compounds in which cations and anions combine to form electrically neutral products.

Isomers : Compounds with the same molecular formula but different structural formula. There are structural isomers and stereoisomers. An example of a structural isomer is a compound with a C=C (double bond) with one halogen attached to each carbon. The halogens could be attached on the same side of the double bond (*cis* isomer) or the halogen atoms could be on opposite sides of the double bond (*trans* isomer). Because the double bond does not rotate, the cis and trans isomer cannot be inter-converted. Structural isomers have different physical properties.

Analogues (Analogs) : Compounds in which one or more individual atoms have been replaced, either with a different atom, or with a different functional group. Another use of the term in chemistry refers to a substance which is similar in structure and/or function to another substance.

Metabolites : Any substance produced by metabolism or by a metabolic process.

Prodrug : A compound resulting from modification of a biologically active compound that will liberate the active form via biotransformation.

Crystal : A crystal is a solid in which the constituent atoms, molecules, or ions are packed in a regularly ordered, repeating pattern extending in all three spatial

dimensions.

Polymorph : Crystals which have the same chemical composition but different internal structure, including different unit cell dimensions and different crystal packing. Problems can occur when another company finds another polymorph that wasn't claimed.

Issues related to polymorphs have been litigated at the Federal Circuit usually after a generic company files an ANDA or NDA. (see *Smithkline v. Apotex,* 403 F.3d 1331 (Fed. Cir. 2005). involves pseudopolymorphs (hydrates) of the pharmaceutical drug.

Note: Jeff Lindeman, Chair of the Chemical Committee, presented at AIPLA meeting – Patent Practice Advanced Chemical and Biotechnology - on the topic of polymorphs in 2006 "Patenting Polymorphs – Claiming Form over Substance."

Solvates : Crystalline solid adducts containing solvent molecules within the crystal structure giving rise to unique differences in physical and pharmaceutical properties of the drugs..

Hydrates :Crystalline solid adducts containing water molecules within the crystal structure.

Prima Facie Case: Wands Factors

•(A) The breadth of the claims;
•(B) The nature of the invention;
•(C) The state of the prior art;
•(D) The level of one skilled in the art;
•(E) The level of predictability in the art;
•(F) The amount of direction provided by the inventor;
•(G) The existence of working examples; and
•(H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Specific technical reasons why the examiner doubts the invention is enabled are always required when establishing a prima facie case of lack of enablement.

The nature of the invention, predictability in the art and state of the prior art

Salts

Preparation of salts of compounds are often routine and predictable in organic chemistry and the pharmaceutical arts.
While salts may be routine to make, the use of derivatives must be considered as well.

Salts of compounds are rarely an enablement issue.

Isomers

The two types of isomers are structural isomers and stereoisomers.
Two compounds are considered structural isomers is they have the *same* molecular formula but *different* connections between atoms (bonding).
Two compounds are considered stereoisomers if they have the *same* molecular formula, the *same* connections between atoms, but *different* arrangements of the atoms in three dimensional space.
Enablement usually resides in the recognition of the isomer and the successful resolution of the racemate.

Analogues

Compounds of this class are usually improved versions of a 'pioneer' drug with pharmacological, pharmacodynamic or biopharmaceutical advantages over the original compound.
Direct analogue design involves straightforward molecular modifications, such as the synthesis of homologues, vinylogues, isosteres, modified ring systems and twin drugs (homodimers).
As a rule, the basic scaffold is conserved or only slightly modified.
Structural analogues may be compounds originally prepared from a novel lead but for which biological assays revealed totally unexpected pharmacological properties.
Observation of a new activity can be purely fortuitous but can also result from planned systematic investigations.
Structural analogues often originate from those serendipitous discoveries that often happen during clinical investigations.
Structural analogues can also result from a systematic application of multi-target screening large series of structurally similar compounds.

Crystalline/Polymorphic Forms of Compounds

Most drugs are used in crystalline form.
The arrangement of molecules in a crystal determine its physical properties.
Physical properties of a drug affect its performance.
Compounds that crystallize as polymorphs exhibit a wide range of different physical and chemical properties including melting point, solubility, density, hardness, crystal shape and spectral properties.
Pharmaceutical processing (temperature, pressure, relative humidity) encountered during drying, granulation , milling and compression affect physical properties of crystals making consistency in products difficult.
Pharmaceutical processing (temperature, pressure, relative humidity) encountered during drying, granulation , milling and compression may affect crystalline structure, making consistency in products based on structural order difficult to determine and

physical properties difficult to maintain.

Characterization of crystals/polymorphs introduce problems which include but are not limited to:
• The degree of disorder introduced into the lattice structure during pharmaceutical preparation of the drug;
• The difficulty in calculating the amount of a single crystal or polymorphic form from a mixture of crystalline forms;
•The challenges to Identify the solid form of the active ingredient in formulated products;
•The transient nature and instability of various polymorphic and crystalline forms of active agents.

Metabolites/ Prodrugs

•Metabolites may be activated in vivo into the active form of a drug by the attachment, rearrangement or removal of some functional group(s) attached to the compounds core. The compound may or may not be modified structurally.
•The biological (in vivo) transformation may facilitate transport to the active site or activation of the drug's therapeutic properties.
•Prodrugs are compounds which are structurally modified which changes the compounds physicochemical properties.
•The conversion of a metabolite or a prodrug may occur via a variety of reactions, the most common being hydrolytic or enzymatic cleavage.

•Many aspects of drug metabolism are of interest to medicinal chemists and should be considered when determining the efficacy of metabolites and prodrugs, such as:
(i) the chemistry and biochemistry of metabolic reactions involved in the conversion of the metabolite or prodrug into the active form of the compound.
(ii) the changes in the compound based upon biotransformation of the metabolite or prodrug.
(iii) predictions of drug metabolism based on quantitative structure metabolism relationships, modeling of enzyme sites and expert systems has advanced substantially in the last decade.
(iv) metabolites and prodrugs are compounds which have been or will be modified which will be subsequently modified in vivo (via metabolic reaction) to provide biologically active compounds.
(v) physiochemical properties of the parent compound are altered to prepare metabolites/prodrugs which influence acidity, basicity, lipophilicity, drug permeability, dosage choice, toxicity, stability and localization of the parent compound.
(vi) the most serious consideration of the metabolite/prodrug is the change to the compound core because a prodrug is most often a new drug and therefore requires extensive and costly studies to determine safety and efficacy.

Solvates/Hydrates

•It has been estimated that approximately one-third of pharmaceutically active substances are capable of forming hydrates.

•Solvates differ in crystal packing and molecular conformation as well as lattice energy.

•Crystalline states of compounds vs. pharmaceutical compositions may require consideration of phase transformation during formulation of compositions.

•Predicting the formation of solvates and hydrates of a compound and the number of molecules of water or solvent incorporated into the crystal lattice of the compound is challenging.

•The reactions and processing involved in the preparation of solvates and hydrates cannot be generalized for a series of related compounds since each solid compound responds uniquely to solvate or hydrate formation

•Pharmaceutical processing (temperature, pressure, relative humidity) encountered during drying, granulation , milling and compression affect crystalline structure, which may make consistency in products based on structural order difficult to determine and physical properties difficult to maintain.

•Each solid compound responds uniquely to the possible formation of solvates and hydrates and generalizations cannot be made for a series of related compounds

Pharmaceutical processing (temperature, pressure, relative humidity) encountered during drying, granulation , milling and compression affect crystalline structure, making consistency in products based on structural order difficult to determine and maintain.

Consideration of hydration/dehydration of active agents requires consideration of conditions during processing, proper packaging, acceptable temperature ranges for shipping and storage, making selection of the specific solid form of the drug critical.

WANDS FACTOR: Direction provided by the inventor and examples.

Questions of enablement may arise when :

•There are no adequate representations advanced in the specification teaching how to make and use the derivatives such as analogues, prodrugs, metabolites, solvates and hydrates.

•The disclosure fails to direct the skilled artisan to relevant prior art teachings which would correlate modification of a compound in a manner which could be extrapolated to compounds set forth in a patent application's claims.

•When the disclosure does not set forth in full, clear and exact terms the identity and location of modifications to the compound.

Patents Issued with Derivatives listed below in the Claims since 2000

5. Enablement in Claims to Therapeutic Treatment

1. *In re Gardner*, 427 F.2d 786, 166 USPQ 138 (C.C.P.A. 1970)[1] (Not Enabled).

Applicants had claimed a pharmaceutical composition having antidepressant activity. The specification, however, lacked the disclosure of the proper dosage, working examples, and an animal model. Rejected by examiner, affirmed by the Board. On appeal Appellants, relying on an affidavit, argued that efficacy in a rat model correlated to antidepressant activity in man, and that the proper dosage would have been within the skill of a pharmacologist.

The court - "In effect, by [claiming therapeutic activity, applicants] are claiming in terms of use. It behooves them, therefore, to disclose how to use, as section 112 ordains . . . ."

2. *In re Jolles*, 628 F.2d 1322, 206 USPQ 885 (C.C.P.A. 1980) (Enabled).

Applicant's claimed pharmaceutical compositions and methods of treating acute myeloblastic leukemia in humans by administering compositions of naphthacene derivatives. The specification discussed the structural similarities of the derivatives to daunorubicin and doxorubicin.

The applicant submitted evidence of: clinical treatment of patients with acute myeloblastic leukemia *and* evidence showing *in vivo* antitumor activity in mice (b) mouse tests on sarcoma tumors and leukemia of eight structurally similar compounds, one of which was the same as tested clinically. The examiner asserted that the invention was not enabled because utility was non-existent, however, the examiner did not provide documentary evidence.

The Board affirmed the examiner without providing documentary evidence.
Evidence showing successful *in vitro* testing supplemented by similar *in vitro* and *in vivo* activities of structurally similar compounds (*Cross*, 753 F.2d at 1051, 224 USPQ at 748); and by evidence showing *in vivo* antitumor activity in mice, combined with a disclosure that the claimed compounds had higher antitumor activity than a related compound known to have antitumor activity (*Brana*, 51 F.3d at 1567, 34 USPQ2d at 1442).

[1] This case was cited in *Smithkline Beecham Corp. v. Apotex Corp.*, 365 F.3d 1306, 70 USPQ2d 1737, 1743 (Fed. Cir. 2004) (The test for indefiniteness ... depends on whether the claim delineates to a skilled artisan the bounds of the invention. . . . Even if a claim is broad enough to embrace undetectable trace amounts of the claimed invention, "[b]readth is not indefiniteness." In re Gardner, 427 F.2d 786, 788 [166 USPQ 138] (CCPA 1970).

3. *In re Bundy*, 642 F.2d 430, 209 USPQ 48 (C.C.P.A. 1981), (Enabled).
Claims drawn to prostaglandin E analogs.

Specification disclosed biological activities of natural PGEs therapeutic uses relying on the biological activities unexpected increase in the analogs' biological activity.

However, there were no working examples included in the specification.
The examiner found a lack of enablement citing a reference stating that "small changes in prostaglandin structure could alter potency or induce diametrically opposed "pharmacological effects."

Held: The evidence of change in pharmacologic activity was related to PGF, not PGE. The discussion of PGE related only to a matter of degree of potency.
Gardener was distinguished due to claims to compounds without recitation of use

Claims to compounds or compositions that do not recite an intended use need only one enabled use. Evidence of unpredictability must be sufficiently related to the claimed invention.

4. Rasmussen v. SmithKline, 413 F.3d 1318, 75 USPQ2d 1297 (Fed. Cir. 2005) (Not Enabled).
Interference appeal in which Rasmussen had lost the interference to SmithKline.
The claims were drawn to methods of treating prostate cancer by administration of a 5aR- inhibiting compound, specifically finasteride.
The Board held that Rasmussen's priority document failed to enable the claimed invention in view of The state of the art , The lack of data to demonstrate the effects of finasteride in treating prostate cancer.

On appeal, Rasmussen argues that:
1. The Board's findings regarding lack of a showing of efficacy are not relevant to a finding of lack of enablement, but pertains only to utility
2. The enablement requirement of Section 112 does not mandate a showing of utility and if it does, the requirement mandates only a showing that it is "not implausible" that the invention will work for its intended purpose
The court disagrees, holding that failure to disclose "how to use" may support a rejection under 35 USC 112, 1st paragraph
"[I]t is proper for the examiner to ask for substantiating evidence unless one with ordinary skill in the art would accept the allegations as obviously correct."
Evidence of unpredictability in the art in the absence of data that resolves the unpredictability is often the basis for a conclusion of lack of enablement.

5 *Impax v. Aventis*, 496 F.Supp.2d 428 (D. Del. 2007) (Not Enabled).

Claims to method of treating ALS by administering riluzole. Impax asserted invalidity based on prior art anticipation of Aventis patent. Aventis argued asserted prior art was not enabling.

Aventis asserted that the patent discloses thousands of formula I compounds and numerous diseases, yielding thousands of possible combinations provides no direction or guidance to arrive at the claimed invention of using riluzole to treat ALS does not disclose any working examples of the claimed invention.

Impax asserted that the patent includes riluzole as a formula I compound suggests that formula I compounds may be used to treat ALS provides some dosage information.

Impax directs the Court to information contained in the patent to suggest that undue experimentation would not be required in human therapy, the compounds according to the invention are especially useful in the treatment and prevention of convulsive phenomena, schizophrenic disorders, and in particular the deficiency forms of schizophrenia, sleep disorders, phenomena linked to cerebral ischaemia and also neurological conditions in which glutamate may be implicated, such as Alzehimer's disease, Huntington's chorea, and ALS.

The District Court
"the compounds of the claimed invention are associated with the treatment of at least eight different diseases, and there is nothing in the patent which would lead one to recognize that any specific compound, let alone riluzole, would be used to treat any specific disease, let alone ALS." that the mere mention of riluzole was insufficient to put one skilled in the art in the possession of the claimed invention as is required to support a conclusion of enablement."

Specification detailing extensive lists of conditions to be treated and compounds to be used, yielding large numbers of possible combinations may suggest lack of enablement of claim to specific combination in the absence of working examples and if evidence of unpredictability exists in the prior art

6. Pharmaceutical Resources v. Roxane Laboratories, Inc., 2007 WL 3151692 (Fed. Cir. 2007) (Not Enabled).

Non-precedential Fed. Cir. opinion affirming the District Court finding that Par's patents were invalid for lack of enablement. Claims to oral pharmaceutical composition of megestrol acetate, choices of specific alcohols and a surfactant.
Claim language did not limit type or amount of surfactant.
Specification stated that invention was not limited to particular surfactants.

Par asserted that broadest reasonable interpretation of claim did not limit type or amount of surfactant.

Par stressed unpredictability in formulation based on type and amount of surfactant during prosecution of patents.

Par's expert testified to unpredictability of formulation with surfactants during previous trial with another litigant

The court held the claims lacked enablement based, in part, on evidence of unpredictability provided previously by Par

The court also considered the breadth of the claims, the presence of working examples and unsupported conclusions in declarations

Evidence of unpredictability presented to support a conclusion of nonobviousness may be appropriate to support a finding of lack of enablement for at least a portion of the scope of the claim

Enablement analysis of therapeutic treatment claims begins with determining the breadth of the claims with regard to the condition being treated.

The compound/composition administered.
Enablement analysis of therapeutic treatment claims continues with determination of the presence of any unpredictability within the state of the art with regard to the condition to be treated.
The compound/composition administered
Enablement analysis of therapeutic treatment claims finishes with the specification by evaluation of the presence or absence of working examples. The evaluation of any other evidence of record, e.g. declarations.
Evidence of unpredictability or predictability may occur in the etiology of the condition/disease. Number/type of other accepted treatments. The presence or absence of art-recognized animal models. Manner of formulation and/or delivery
The Examiner must weigh the evidence and provide the rationale.

No per se rules!

6. Rejoinder Practice

What is "Rejoinder"?
o The process of withdrawing a restriction requirement between an allowable elected invention and a non-elected invention when all claims to a non-elected invention depend from or otherwise require all the limitations of an allowable claim. Rejoined claims must still be fully examined. MPEP 821.04.

Criteria for Distinct Inventions

- For restriction and examination, distinctness between related inventions requires that at least one invention would not have been obvious over the other.

- For allowance, distinction between related inventions requires that claims to the non-elected inventions are distinct from the elected, allowable invention.

- Rejoinder within the same statutory category of invention
- § 1.141 Different inventions in one national application.
- Two or more independent and distinct inventions may not be claimed in one national application, except that more than one species of an invention, not to exceed a reasonable number, may be specifically claimed in different claims in one national application, provided the application also includes an allowable claim generic to all the claimed species and all the claims to species in excess of one are written in dependent form (§ 1.75) or otherwise include all the limitations of the generic claim.

- Rejoinder of Processes with Allowable Product
- In re Ochiai, 71 F.3d 1565, 37 USPQ2d 1127 (Fed. Cir. 1995) and In re Brouwer, 77 F.3d 422, 37 USPQ2d 1663 (Fed. Cir. 1996) addressed the issue of whether an otherwise conventional process could be patented if it were limited to making or using a nonobvious product (*different statutory classes*).

- In both cases, the Federal Circuit held that the use of per se rules is improper in applying the test for obviousness under 35 U.S.C. 103. Rather, 35 U.S.C. 103 requires a highly fact-dependent analysis involving taking the claimed subject matter as a whole and comparing it to the prior art.

- "A process yielding a novel and nonobvious product may nonetheless be obvious; conversely, a process yielding a well-known product may yet be nonobvious." TorPharm, Inc. v. Ranbaxy Pharmaceuticals, Inc., 336 F.3d 1322, 1327, 67 USPQ2d 1511, 1514 (Fed. Cir. 2003).

- MPEP 2121

Eligibility for Rejoinder
- In order to be eligible for rejoinder, a claim to a non-elected invention must depend from or otherwise require all the limitations of an allowable claim.
- Claims that do not require all the limitations of an allowable claim remain withdrawn from consideration.

Allowability of Rejoined Claims
- Rejoined claims must be fully examined for patentability in accordance with 37 CFR 1.104.
- Double Patenting Concerns, MPEP 821.04
- The requirement for restriction between rejoined inventions must be withdrawn.

o Any claims presented in a continuation or divisional application that are anticipated by, or rendered obvious over, the claims of the parent application may be subject to a non-statutory double patenting rejection if the restriction requirement has been withdrawn in the parent application. In re Ziegler, 443 F.2d 1211, 1215, 170 USPQ 129, 131-32 (CCPA 1971). See also MPEP § 804.01.

Making Second Action Final

o If rejoinder occurs after the first Office action on the merits, and if any of the rejoined claims are unpatentable, e.g., if a rejection under 35 U.S.C. 112, first paragraph is made, then the next Office action may be made final where the new ground of rejection was necessitated by applicant's amendment.

o MPEP § 706.07(a).

o Rejoinder Between Related Inventions in the *same* Statutory Category

o Rejoining claims to a combination that requires all the limitations of an allowable subcombination

o Rejoining claims to species which are encompassed by an allowable generic claim

o MPEP 821.04(a)

o An amendment presenting additional claims that depend from or otherwise require all the limitations of an allowable claim will be entered as a matter of right if the amendment is presented prior to final rejection or allowance, whichever is earlier.

o Amendments submitted after final rejection are governed by 37 CFR 1.116; amendments submitted after allowance are governed by 37 CFR 1.312.

o Rejoinder within same statutory category of invention

o Once the elected invention is found to be allowable, an examiner should use FP 8.45, 8.49, or 8.50 to advise applicant of the status of the other inventions.

o If non-elected claims which depended from or otherwise required all the limitations of an allowable claim were cancelled by applicant and may be reinstated by submitting the claims in an amendment, the examiner should use FP 8.46, 8.47, or 8.47.01 to inform applicant.

o Note that each additional invention is considered separately.

o When claims to one non-elected invention depend from or otherwise require all the limitations of an allowable claim, and claims to another non-elected invention do not, applicant must be advised as to which claims have been rejoined and which claims remain withdrawn from further consideration.

Rejoinder within *same statutory category* of invention

o Where the application claims an allowable invention and discloses but does not claim an additional invention that depends on or otherwise requires all the limitations of the allowable claim, applicant may add claims directed to such additional invention by way of amendment pursuant to 37 CFR 1.121.

o Amendments submitted after allowance are governed by 37 CFR 1.312.

o Amendments submitted after final rejection are governed by 37 CFR 1.116.

- Rejoining a process of making a product which requires an allowable product
- Rejoining a process of using a product which requires an allowable product
  Applicant must elect the product invention.
- Non-elected Products are not considered for rejoinder upon allowance of a process invention.
- Allowability of a process invention does not correlate with novelty or nonobviousness of a product made by or used in the process. See MPEP 821.04(b).
- Where the application as originally filed discloses the product and the process for making and/or using the product, and only claims directed to the product are presented for examination, applicant may present claims directed to the process of making and/or using the allowable product.

Rejoinder Between Related Inventions of <u>different</u> Statutory Categories (i.e., "Products" and "Processes").

To expedite prosecution, applicants are encouraged to present such process claims, preferably as dependent claims, in the application at an early stage of prosecution.

- Process claims which depend from or otherwise require all the limitations of the patentable product will be entered as a matter of right if the amendment is presented prior to final rejection or allowance, whichever is earlier.
- If an amendment adds claims to a process invention, and the amendment includes process claims which do not depend from or otherwise require all the limitations of an allowable product, all claims directed to that newly added invention may be withdrawn from consideration, via an election by original presentation.

MPEP 821.03

- If an amendment after final rejection that otherwise complies with the requirements of 37 CFR 1.116 would place all the elected product claim(s) in condition for allowance and thereby require rejoinder of process claims that raise new issues requiring further consideration (e.g., issues under 35 U.S.C. 101 or 112, first paragraph), the amendment could be denied entry.

- Before mailing an advisory action in the above situation, it is recommended that applicant be called and given the opportunity to cancel the process claims to place the application in condition for allowance with the allowable product claims, or to file an RCE to continue prosecution of the process claims in the same application as the product claims.

For 371 Applications

- If the 1st claimed product invention does not make a contribution over the prior art (there are reference(s) anticipating and rendering obvious the product as broadly claimed), then it would be proper to group all the methods separate from the product.
- 37 CFR 1.475(d)
- For 371 Applications

- If the 1st claimed product invention makes a contribution over the prior art (novel and unobvious), then it would be grouped with and examined with 1st claimed method of making the product and 1st claimed method of using the product. The second and subsequent methods of making or using the product may be withdrawn for lacking unity of invention.
- 37 CFR 1.475(d)

For 371 Applications

- When all the claims to the 1st claimed product invention all allowable, then the lack of unity determination would be withdrawn between the elected invention and any method inventions in which all claims depended from or otherwise require all the limitations of an allowable product claim.

Rejoinder of Process Claims Requiring an Allowable Product, MPEP 821.04(b)

- Rejoinder may be appropriate when claims to an elected product are allowable and ALL claims to a non-elected process of making and/or using the product depend from or otherwise require all limitations of the allowable product claim
- In order to retain the right to rejoinder, applicant is advised that the claims to the non-elected invention(s) should be amended during prosecution to require the limitations of the elected invention.
- Failure to do so may result in a loss of the right to rejoinder.
- If applicant cancels all claims to a non-elected process invention before rejoinder occurs, the examiner should not withdraw the restriction requirement between the product and process.
- This will preserve the applicant's rights under 35 USC 121 to file divisional applications without being subject to non-statutory double patenting rejections.

Double Patenting Between Product and Process Inventions

- Where applicant voluntarily presents claims to the product and process in separate applications (i.e., no restriction requirement was made by the Office), and one of the applications issues as a patent, the remaining application may be rejected under the doctrine of obviousness-type double patenting.
- Applicant may overcome the rejection by the filing of a terminal disclaimer where appropriate.
- Double Patenting Between Product and Process Inventions
- If copending applications separately present product and process claims, provisional obviousness-type double patenting rejections should be made where appropriate.
- However, once a determination as to the patentability of the product has been reached any process claim directed to making or using an allowable product should not be rejected over prior art without consultation with a Technology Center Director.

EXAMPLES

Same Statutory category

Example 1: Genus/Species Claim (Rejoinder)

Claim 1. (Original) A method of reducing pain by administering to a patient a composition comprising a compound having Formula I and a botanical extract. *[Linking claim generic to species I, II and III]*

Claim 2. (Original) The method of claim 1, wherein the botanical extract is an aqueous extract of *Piper methysticum* (kava-kava). *[Species I]*

Claim 3. (Withdrawn) The method of *claim 1*, wherein the botanical extract is an aqueous extract of *Vitis vinefera* (grape) seeds. *[Species II]*

Claim 4. (Withdrawn) The method of *claim 1*, wherein the botanical extract is an alcohol extract of *Echinacea purpurea. [Species III]*

Rejoinder

- The examiner required an election of species I, II, or III. Species I was elected. Claims 3 and 4 are initially withdrawn from examination. Claims 1 and 2 are allowable.
- Because all claims to the elected invention are in condition for allowance, the examiner should withdraw the election of species requirement between Species I, II and III.
- The inventions defined by claim 3 and 4 should be rejoined with the invention of claim 1 because claim 1 is *generic* to Species I, II and III.

Example 2: Genus/Species of Example 1. Genus, Linking Claim, is Not Allowable (No Rejoinder).

- Species I was elected. Claim 2 is allowable.
- Linking claim 1 is not allowable.
- Because not all of the claims directed to the elected invention are in condition for allowance, rejoinder is not required.
- Because the linking claim is rejected, the examiner is not required to examine second or subsequence species recited in claims 3 or 4.

Example 3: Genus/Species claims of Example 1 plus Claim 5 (new linking claim) (No rejoinder)

Claim 1. (Original) A method of reducing pain by administering to a patient a composition comprising a compound having Formula I and a botanical extract. *[Linking claim generic to species I, II and III]*

Claim 2. (Original) The method of claim 1, wherein the botanical extract is an aqueous extract of *Piper methysticum* (kava-kava). *[Species I]*

Claim 3. (Withdrawn) The method of claim 1, wherein the botanical extract is an aqueous extract of *Vitis vinefera* (grape) seeds. *[Species II]*

Claim 4. (Withdrawn) The method of claim 1, wherein the botanical extract is an alcohol extract of *Echinacea purpurea*. *[Species III]*

Claim 5. (Original) A method of curing cancer by administering to a patient a composition comprising a compound having Formula I and a botanical extract. *[Linking claim generic to species I, II and III]*

- An election of species I, II, or III is required; species I was elected.
- Claims 1, 2 and 5 read upon the elected invention.
- Claims *1 and 2 are allowable.*
- Claims 3 and 4 recite all the limitations of allowable claims.
- However, *claim 5 is rejected* under 112, 1st because the specification has not enabled "curing cancer."
- Because not all claims directed to the elected invention are in condition for allowance, the examiner is not required to rejoin claims 3 and 4.

Different Statutory Categories

Example 4: Two statutory classes (Rejoined).
Claim 1. (Original) A composition comprising an alcohol extract of *Vitis vinefera* (grape) seeds.

Claim 2. (Original) A method of treating diabetes by administering composition comprising an alcohol extract of *Vitis vinefera* (grape) seeds.

Claim 3. (Original) A method of treating diabetes by administering composition comprising an extract of *Vitis vinefera* (grape) seeds and an alcohol extract of *Piper methysticum* (kava-kava).

- The examiner required restriction between product (composition) Invention Group I (claim 1) and process invention Group 2 (claim 2 and 3).

- Applicant elected Group I and amended claim 3 to the following:

Claim 3. (Amended) A method of treating diabetes by administering composition comprising an <u>alcohol</u> extract of *Vitis vinefera* (grape) seeds and an alcohol extract of *Piper methysticum* (kava-kava).

o After claim 1 is determined to be allowable, the examiner should withdraw the restriction requirement.
o Claims 2 and 3 should be rejoined with claim 1 (see MPEP 821.04(b), FP 8.42, 8.43)).

Example 5: Two statutory classes (Not Rejoined).

Claim 1. (Original) A composition comprising an alcohol extract of *Vitis vinefera* (grape) seeds.

Claim 2. (Original) A method of treating diabetes by administering composition comprising an alcohol extract of *Vitis vinefera* (grape) seeds.

Claim 3. (Original) A method of treating diabetes by administering composition comprising an extract of *Vitis vinefera* (grape) seeds and an alcohol extract of *Piper methysticum* (kava-kava).

o After claim 1 is determined to be allowable, the examiner should *NOT* withdraw the restriction requirement.
o Claim 2 should not be rejoined.
o Applicants may file claims 2 and 3 in a divisional application without being subject to double patenting rejections.

Example 6: Two statutory classes (Not Rejoined)

Claim 1. (Original) A composition comprising an alcohol extract of *Vitis vinefera* (grape) seeds.

Claim 2. (Original) A method of treating diabetes by administering composition comprising an alcohol extract of *Vitis vinefera* (grape) seeds.

o The examiner required restriction between the product of Group I (claim 1) and Process of Group II (claim 2).
o Applicants elected to Process of Group II.
o Examiner finds *Group II allowable*.
o The examiner is not obligated to rejoin a product with an allowable process.
o Patentability of a process does not correlate with novelty and non-obviousness of a product used in that that process.
o Importance of a Clear Record
o A clear and detailed record of the restriction requirement provides a clear demarcation between restricted inventions.
o Applicants have adequate notice regarding the inventions subject to restriction.
o If applicants seek relief from a restriction requirement by petition, a clear record simplifies the petition decision process.

o An examiner/court can determine whether inventions claimed in a continuing application are consonant with the restriction requirement and therefore subject to the prohibition against double patenting rejections under 35 U.S.C. 121.

Geneva Pharms. Inc. v. GlaxoSmithKline PLC, 68 USPQ2d 1865, 1871 (Fed. Cir. 2003).

Switching Inventions after Election

o Applicant is generally *not* permitted to switch to claiming a different invention after a first action on the merits.

o Cancellation of all claims drawn to an elected invention and presentation of claims drawn to a non-elected invention is non-responsive. Applicant given one month or 30 days to file a responsive amendment.

o An RCE may not be used as a matter of right to switch to an invention which is independent or distinct from the invention examined previously.

o MPEP 819 and 821.03

Constructive Election by original presentation.

o Claims added *after* an Office action should be withdrawn as non-elected by "original presentation" ONLY if those claims are drawn to an invention that is independent or distinct from the invention examined on the merits.

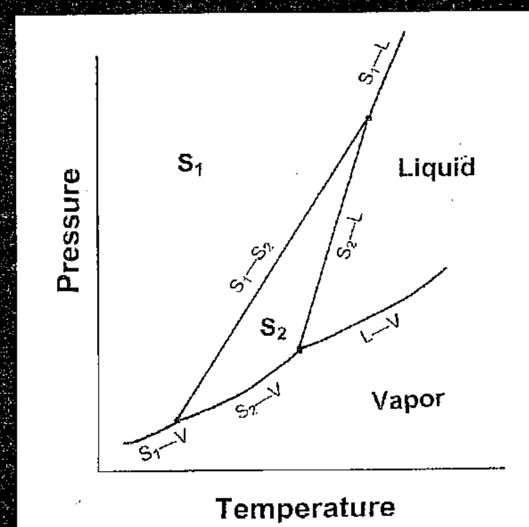
o Where applicant presents claims that could not have been restricted from the claims drawn to other elected invention had they been presented earlier, the newly added claims (if entered) must be examined on the merits.

Future Meeting Topics

- At the end of each meeting, the Group Director solicits topics for future meetings; every attendee has the opportunity to request a future topics. If there are topics that you would like the UPSTO to address, please email John Calve (JCalve_Patents@verizon.net), (202)-483-6482 of the Biotechnology Committee. If you have a specific question about an upcoming topic that you would like me to ask the presenter, please let me know.
- Alternatively, Cecilia Tsang (571-272-0562; cecilia.tsang@uspto.gov) or Group Directors of Technology Center 1600 can be emailed: George Elliot, John LeGuyader and Bruce Kisliuk.
- The next meeting is expected to occur in March of 2008.

Notes prepared by John N. Calve, Esq., Biotechnology Subcommittee on USPTO issues.

Polymorphism in Pharmaceutical Solids



edited by
Harry G. Brittain

5

Generation of Polymorphs, Hydrates, Solvates, and Amorphous Solids

J. Keith Guillory

The University of Iowa
Iowa City, Iowa

I. METHODS EMPLOYED TO OBTAIN UNIQUE POLYMORPHIC FORMS

Organic medicinal agents that can exist in two or more solid phases often can provide some distinct advantages in particular applications. The metastable solid may be preferred in those instances where absorption of the drug is dissolution rate dependent. The stable phase may be less susceptible to chemical decomposition and may be the only form that can be used in suspension formulations. Often a metastable polymorph can be used in capsules or for tableting, and the thermodynamically stable form for suspensions. Factors related to processing, such as powder flow characteristics, compressibility, filterability, or hygroscopicity, may dictate the use of one polymorph in preference to another. In other cases, a particular form may be selected because of the high reproducibility associated with its isolation in the synthetic procedure.

It is essential to ascertain whether the crystalline material that results from a synthetic procedure is thermodynamically stable before conducting pivotal trials, since a more stable form may be obtained subsequently, and it may be impossible to produce the metastable form in future syntheses. Conversion from one polymorph to another can occur during processing or upon storage. An additional incentive for

isolating and identifying polymorphs that provides certain advantages is the availability of subsidiary patents for desirable polymorphic forms, or for retaining a competitive edge through unpublished knowledge. In 1990 Byrn and Pfeiffer found more than 350 patents on crystal forms granted on the basis of an advantage in terms of stability, formulation, solubility, bioavailability, ease of purification, preparation or synthesis, hygroscopicity, recovery, or prevention of precipitation [1].

One question that is likely to arise during the registration process is "What assurance can be provided that no other crystalline forms of this compound exist?" It is incumbent on the manufacturer of a new drug substance to show that due diligence has been employed to isolate and characterize the various solid-state forms of a new chemical entity. This may seem to be a daunting task, particularly in light of the widely quoted statement by Walter C. McCrone [2] that "Those who study polymorphism are rapidly reaching the conclusion that all compounds, organic and inorganic, can crystallize in different crystal forms or polymorphs. In fact, the more diligently any system is studied the larger the number of polymorphs discovered." On the other hand, one can take comfort from the fact that some important pharmaceuticals have been in use for many years and have, at least until now, exhibited only one stable form. Indeed, it seems to this author that there must be particular bonding arrangements of some molecules that are so favorable energetically as to make alternate arrangements unstable or nonisolatable.

In the future, computer programs using force-field optimization should be perfected to the point where it will be possible to predict, with confidence, that a particular crystalline packing arrangement is the most stable that is likely to be found. These programs also may make it possible to predict how many alternate arrangements having somewhat higher energy can potentially be isolated [3,4]. Until that time, the developmental scientist is handicapped in attempting to predict how many solid forms of a drug are likely to be found. The situation is further complicated by the phenomenon of "disappearing polymorphs" [5], or metastable crystal forms that seem to disappear in favor of more stable ones.

Some polymorphs can be detected, but not isolated. Hot stage microscopy has been used extensively to study polymorphic transfor-

mations. The microscopist can detect numerous polymorphic transformations, but the individual polymorphs often prove to be so unstable that they cannot be isolated by the usual methods. An excellent example of this is the work of Grießer and Burger on etofylline [6]. These authors identified five polymorphic forms by thermomicroscopy, but only stable Modification I could be obtained by recrystallization, even when seed crystals from the hot stage were used. Similarly, Kuhnert-Brandstätter, Burger, and Völlenklee [7] described six polymorphic forms of piracetam, only three of which could be obtained by solvent crystallization. All the others were found only by crystallization from the melt. What, then, is a careful investigator to do?

In this chapter, the various methods used to isolate polymorphs, hydrates, and solvates will be described. As Bernstein [8] has observed, "The conditions under which different polymorphs are obtained exclusively or together also can provide very useful information about the relative stability of different phases and the methods and techniques that might be necessary to obtain similar structures of different chemical systems." In this context, it is hoped that the following information will prove useful in devising a "screening" protocol for the preparation of the various solid state forms of pharmaceuticals. While one cannot be absolutely certain that no additional forms will be identified in the future, this approach should provide some assurance that "due diligence" has been exercised to isolate and identify crystalline forms that are likely to arise during the normal course of drug development and storage.

A. Sublimation

On heating, approximately two-thirds of all organic compounds are converted partially from the solid to the gaseous state and back to solid, i.e., they sublime [9]. While strictly speaking the term sublimation refers only to the phase change from solid to vapor without the intervention of the liquid phase, it is often found that crystals are formed on cooler surfaces in close proximity to the melt of organic compounds when no crystals were formed at temperatures below the melting point. The most comprehensive information concerning sublimation temperatures of compounds of pharmaceutical interest can be found in tables

in the textbook of Kuhnert-Brandstätter [9]. While the information in these tables is designed primarily for the microscopic examination of compounds, it is also possible to utilize it to determine which compounds might be susceptible to the application of techniques (such as vacuum sublimation) that can be carried out on larger scales and at lower temperatures.

The sublimation temperature and the distance of the collecting surface from the material undergoing sublimation have a great influence on the form and size of the crystals produced. The occurrence of polymorphic modifications depends on the temperature of sublimation. In general, it may be assumed that unstable crystals form preferentially at lower temperatures, while at higher temperatures stable forms are to be expected. Nevertheless, mixtures consisting of several modifications are frequently found together. This is the case for barbital and for estradiol benzoate. It should be obvious that the sublimation technique is applicable only to those compounds that are thermally stable.

A simple test can be used to determine if a material sublimes. A small quantity (10–20 mg) of the solid is placed in a petri dish that is covered with an inverted watch glass. The petri dish is heated gently on a hot plate and the watch glass is observed to determine if crystals are growing on it. According to McCrone [2], one of the best methods for obtaining a good sublimate is to spread the material thinly over a portion of a half-slide, cover with a large cover glass, and heat slowly using a Kofler block. When the sublimate is well formed, the cover glass is removed to a clean slide for examination. It is also possible to form good crystals by sublimation from one microscope slide to a second held above it, with the upper slide also being heated so that its temperature is only slightly below that of the lower slide. Cooling of the cover slip by placing drops of various low-boiling solvents on the top surface will cause condensation of the more unstable forms, the lower temperatures leading to the most unstable forms. On a larger scale, a glass cold finger or a commercial sublimator can be employed. Once crystals of various modifications have been obtained, they can be used as seeds for the solution phase crystallization of larger quantities.

Form I of 9,10-anthraquinone-2-carboxylic acid was obtained as needle-like crystals upon sublimation at temperatures exceeding 250°C [10]. Fokkens et al. have used sublimation to purify theophylline for

vapor pressure studies [11]. Sakiyama and Imamura found that stable phases of both 1,3-dimethyluracil and malonamide could be prepared by vacuum sublimation [12].

B. Crystallization from a Single Solvent

Slow solvent evaporation is a valuable method for producing crystals. Solutions of the material being crystallized, preferably saturated or nearly so, are filtered to remove most nuclei and then left undisturbed for a reasonable period of time. The rate of evaporation is adjusted by covering the solution with aluminum foil or Parafilm® containing a few small holes. For a solvent to be useful for recrystallization purposes, the solubility of the solute should be on the order of 5–200 mg/mL at room temperature. If the solubility exceeds 200 mg/mL, the viscosity of the solution will be high, and a glassy product is likely to be obtained. A useful preliminary test can be performed on 25–50 mg of sample, adding a few (5–10) drops of solvent. If all the solid dissolves, the solvent will not be useful for recrystallization purposes. Similarly, highly viscous solvents, and those having low vapor pressures (such as glycerol or dimethylsulfoxide) are not usually conducive to efficient crystallization, filtration, and washing operations. The solvents selected for recrystallization should include any with which the compound will come into contact during synthesis, purification, and processing, as well as solvents having a range of boiling points and polarities. Examples of solvents routinely used for such work are listed in Table 1 together with their boiling points.

The process of solution mediated transformation can be considered the result of two separate events, (a) dissolution of the initial phase, and (b) nucleation/growth of the final, stable phase. If crystals do not grow as expected from a saturated solution, the interior of the vessel can be scratched with a glass rod to induce crystallization by distributing nuclei throughout the solution. Alternatively, crystallization may be promoted by adding nuclei, such as seed crystals of the same material. For example, Suzuki showed that the α-form of inosine could be obtained by crystallization from water, whereas isolation of the β-form required that seeds of the β-form be used [13].

If two polymorphs differ in their melting point by 25–50°C, for monotropic polymorphs the lower melting, more soluble, form will be difficult to crystallize. The smaller the difference between the two melting points, the more easily unstable or metastable forms can be obtained.

Table 1 Solvents Often Used in the Preparation of Polymorphs

Solvent	Boiling point (°C)
Dimethylformamide	153
Acetic acid	118
Water	100
1-Propanol	97
2-Propanol	83
Acetonitrile	82
2-Butanone	80
Ethyl acetate	77
Ethanol	78
Isopropyl ether	68
Hexane	69
Methanol	65
Acetone	57
Methylene chloride	40
Diethyl ether	35

A commonly used crystallization method involves controlled temperature change. Slow cooling of a hot, saturated solution can be effective in producing crystals if the compound is more soluble at higher temperatures; alternatively, slow warming can be applied if the compound is less soluble at higher temperatures. Sometimes it is preferable to heat the solution to boiling, filter to remove excess solute, then quench cool using an ice bath or even a dry ice–acetone bath. High boiling solvents can be useful to produce metastable polymorphs. McCrone [2] describes the use of high boiling solvents such as benzyl alcohol or nitrobenzene for recrystallization on a hot stage. Behme et al. [14] showed that when buspirone hydrochloride is crystallized above 95°C the higher melting form is obtained; below 95°C the lower

melting form is obtained. Thus the lower melting polymorph could be converted to the higher melting polymorph by recrystallizing from xylene (boiling point 137–140°C).

To understand how temperature influences the composition of crystals that form, it is useful to examine typical solubility–temperature diagrams for substances exhibiting monotropic and enantiotropic behavior [15]. In Fig. 1a, Form II, having the lower solubility, is more stable than Form I. These two noninterchangeable polymorphs are monotropic over the entire temperature range shown. For indomethacin, such a relationship exists between Forms I and II, and between Forms II and III.

In Fig. 1b, Form II is stable at temperatures below the transition temperature T_t, and Form I is stable above T_t. At the transition temperature the two forms have the same solubility, and reversible transformation between enantiotropic Forms I and II can be achieved by temperature manipulation. The relative solubility of two polymorphs is a

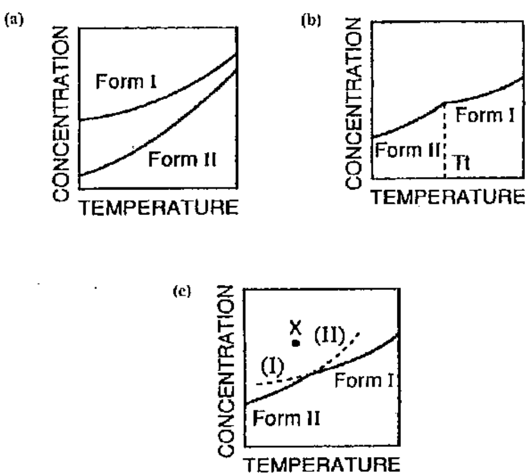


Fig. 1 Solubility curves exhibiting (a) monotropy, (b) enantiotropy, and (c) enantiotropy with metastable phases. (Reprinted with permission of the copyright holder [15].)

convenient measure of their relative free energies. The polymorph having the lower solubility is the more thermodynamically stable form, i.e., the form with the lower free energy at the temperature of the solubility measurement. At room temperature, carbamazepine Form I (m.p. 189°C) is more soluble than is Form III (m.p. 174°C), so the form with the higher melting point is more soluble. The polymorphs are enantiotropic with respect to each other [16].

There are situations in which kinetic factors can for a time override thermodynamic considerations. Figure 1c depicts the intervention of metastable phases (the broken line extensions to the two solubility curves). If a solution of composition and temperature represented by point X (supersaturated with respect to both I and II) is allowed to crystallize, it would not be unusual if the metastable Form I crystallized out first even though the temperature would suggest that Form II would be the more stable (i.e., less soluble) form. This is an extension of Ostwald's law of stages [17], which states that "when leaving an unstable state, a system does not seek out the most stable state, rather the nearest metastable state which can be reached with loss of free energy." This form then transforms to the next most soluble form through a process of dissolution and crystallization. Crystallization of Form I when Form II is more stable would be expected if Form I had the faster nucleation and/or crystal growth rate. However, if the crystals of Form I were kept in contact with the mother liquor, transformation could occur as the more soluble Form I crystals dissolve and the less soluble Form II crystals nucleate and grow. For crystals that exhibit this type of behavior, it is important to isolate the metastable crystals from the solvent by rapid filtration so that phase transformation will not occur.

In the general case, if there are any other polymorphic forms with solubilities below that of Form II, the above-described process will continue between each successive pair of forms until the system finally contains only the most stable (the least soluble) form. The implication of this hypothesis is that, by controlling supersaturation and by harvesting crystals at an appropriate time, it should be possible to isolate the different polymorphic forms. Furthermore, the theory predicts that at equilibrium the product of any crystallization experiment must be the stable form, regardless of the solvent system. It is apparent, however,

from the literature that for some solutes it is the choice of solvent rather than the effects of supersaturation that determines the form that crystallizes [18].

Crystallization of mannitol as a single solute was found to be influenced by both the initial mannitol concentration and by the rate of freezing [19]. In the range of 2.5% to 15%, the δ-polymorph is favored by higher concentrations, whereas the β-polymorph is favored at lower concentrations. At constant mannitol concentration (10%), the α-polymorph is favored by a slow freezing rate, whereas the δ-polymorph is favored by a fast freezing rate.

Kaneko et al. [20] observed that both the cooling rate and the initial concentration of stearic acid in *n*-hexane solutions influenced the proportion of polymorphs A, B, C, and E that could be isolated. Garti et al. [21] reported that for stearic acid polymorphs crystallized from various organic solvents, a correlation was observed between the polymorph isolated and the extent of solvent–solute interaction.

The reason for using crystallization solvents having varying polarities is that molecules in solution often tend to form different types of hydrogen-bonded aggregates, and that these aggregate precursors are related to the crystal structures that develop in the supersaturated solution [22]. Crystal structure analysis of acetanilide shows that a hydrogen-bonded chain of molecules is aligned along the needle axis of the crystals. This pattern is characteristic of secondary amides that crystallize in a trans conformation so that the carbonyl acceptor group and the –NH hydrogen bond donor are anti to one another. The morphology of acetanilide crystals can be controlled by choosing solvents that promote or inhibit the formation of this hydrogen-bond chain. Hydrophobic solvents such as benzene and carbon tetrachloride will not participate in hydrogen-bond formation, so they will induce the formation of rapidly growing chains of hydrogen-bonded amides. Crystals grown by evaporation methods from benzene or carbon tetrachloride are long needles. Solvents that are proton donors or proton acceptors inhibit chain formation by competing with amide molecules for hydrogen-bonding sites. Thus acetone inhibits chain growth at the –NH end, and methanol inhibits chain growth at the carbonyl end of the chain. Both solvents encourage the formation of rod-like acetanilide crystals, while

mixtures of benzene and acetone give hybrid crystals that are rod-shaped, with fine needles growing on the ends [23].

Some solvents favor the crystallization of a particular form or forms because they selectively adsorb to certain faces of some polymorphs, thereby either inhibiting their nucleation or retarding their growth to the advantage of others. Among the factors affecting the types of crystal formed are (a) the solvent composition or polarity, (b) the concentration or degree of supersaturation, (c) the temperature, including cooling rate and the cooling profile, (d) additives, (e) the presence of seeds, (f) pH, especially for salt crystallization, and (g) agitation [22].

Martínez-Ohárriz et al. [24] found that Form III of diflunisal is obtained from polar solvents, whereas Forms I and IV are obtained from nonpolar solvents. Likewise, Wu et al. [25] observed that when moricizine hydrochloride is recrystallized from relatively polar solvents (ethanol, acetone, and acetonitrile), Form I is obtained, whereas nonpolar solvents (methylene chloride or methylene chloride/ethyl acetate) yield Form II.

In determining what solvents to use for crystallization, one should be careful to select those likely to be encountered during formulation and processing. Typically these are water, methanol, ethanol, propanol, isopropanol, acetone, acetonitrile, ethyl acetate, and hexane. Matsuda employed 27 organic solvents to prepare two polymorphs and six solvates of piretanide [26].

According to McCrone [27], in a poor solvent the rate of transformation of a metastable to a more stable polymorph is slower. Hence a metastable form once crystallized can be isolated and dried before it is converted to a more stable phase by solution phase mediated transformation. In some systems the metastable form is extremely unstable and may be prepared only with more extreme supercooling. This is usually performed on a very small scale with high boiling liquids so that a saturated solution at a high temperature that is suddenly cooled to room temperature will achieve a high degree of supersaturation [28].

There are many examples in the literature of the use of single solvents as crystallization screens. Slow crystallization from acetone, acetonitrile, alcohols, or mixtures of solvents yields the Form A of

fosinopril sodium, but rapid drying of a solution of this compound yields Form B, sometimes contaminated with a small amount of Form A [29]. A rotary evaporator can be used to maintain a solution at the appropriate temperature as solvent is being removed.

Form I of dehydroepiandrosterone was obtained by recrystallization from warm ethyl acetate, acetone, acetonitrile, or 2-propanol. Form II was obtained by rapid evaporation, using a vacuum from solutions in dioxane, tetrahydrofuran, or chloroform (which are higher boiling, less polar solvents) [30].

C. Evaporation from a Binary Mixture of Solvents

If single-solvent solutions do not yield the desired phase, mixtures of solvents can be tried. Multicomponent solvent evaporation methods depend on the difference in the solubility of the solute in various solvents. In this approach, a second solvent in which the solute is sparingly soluble is added to a saturated solution of the compound in a good solvent. Often a solvent system is selected in which the solute is more soluble in the component with the higher vapor pressure. As the solution evaporates, the volume of the solution is reduced and, because the solvents evaporate at different rates, the composition of the solvent mixture changes.

Occasionally, crystals are obtained by heating the solid in one solvent and then pouring the solution into another solvent or over cracked ice. Otsuka et al. [31] obtained phenobarbital Form B by adding dropwise a saturated solution of the compound in methanol to water at room temperature. Form E was obtained by the same technique, but by using a saturated solution of phenobarbital in dioxane.

Kitamura et al. have shown that the fraction of Form A of *L*-histidine decreases quickly when the volume fraction of ethanol in an ethanol–water solvent system increases above 0.2, and that pure Form B is obtained at a 0.4 volume fraction of ethanol [32]. The transformation rate for conversion of Form B to Form A decreases with ethanol concentration. The authors postulated that the concentration of the conformer that corresponds to Form A decreases more with ethanol concentration than that of Form B, and so the growth rate of Form A will also decrease.

An example of precipitation in the presence of a second solvent is seen in the case of indomethacin. The γ-crystal form of indomethacin can be obtained by recrystallization from ethyl ether at room temperature, but the α-form is prepared by dissolution in methanol and precipitation with water at room temperature [33]. Precipitation can also result from the addition of a less polar solvent. Form II of midodrine hydrochloride, metastable with respect to Form I, can be prepared by precipitation from a methanolic solution by means of a less polar solvent such as ethyl acetate or dichloromethane [34].

In Fig. 2, three crystalline modifications of thalidomide are illustrated. These were obtained by solvent recrystallization techniques and differ both in crystal habit and in crystal structure. Two of the forms were obtained from a single solvent, and one from a binary mixture.

D. Vapor Diffusion

In the vapor diffusion method, a solution of the solute in a good solvent is placed in a small, open container that is then stored in a larger vessel containing a small amount of a miscible, volatile nonsolvent. The larger vessel (often a desiccator) is then tightly closed. As solvent equilibrium is approached, the nonsolvent diffuses through the vapor phase into the solution, and saturation or supersaturation is achieved. The solubility of the compound in a precipitant used in a two-solvent crystallization method such as vapor diffusion should be as low as possible (much less than 1 mg/mL), and the precipitant (the solvent in which the compound is poorly soluble) should be miscible with the solvent and the saturated solution. The most frequent application of this technique is in the preparation of single crystals for crystallographic analysis. An illustration of the technique is provided in Fig. 3 [35].

E. Thermal Treatment

Frequently when using differential scanning calorimetry as an analysis technique, one can observe an endothermic peak corresponding to a phase transition, followed by a second endothermic peak corresponding to melting. Sometimes there is an exothermic peak between the two endotherms, representing a crystallization step. In these cases it is often

A)

B)

C)

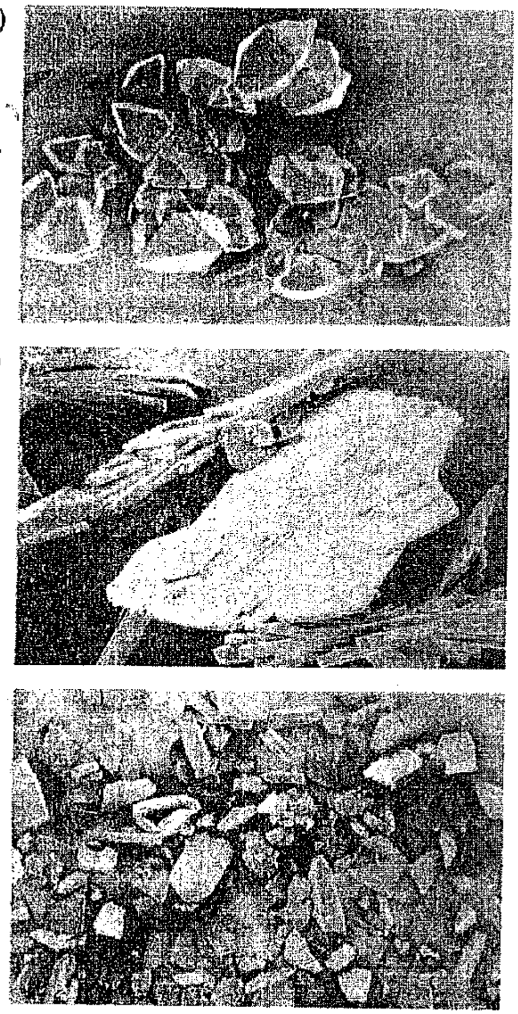


Fig. 2 Three crystalline modifications of thalidomide obtained by solvent recrystallization. (A) Form I obtained as bipyramids by slow crystallization of thalidomide in 1:1 dimethylformamide:ethanol at room temperature. (B) Form II obtained by immersing a saturated solution of thalidomide in acetonitrile in an ice bath. (C) Form III prepared as tabular crystals from a solution in boiling 1,4-dioxane, filtered, then allowed to cool to room temperature. (Photomicrographs courtesy of Dr. S. A. Botha, the University of Iowa.)

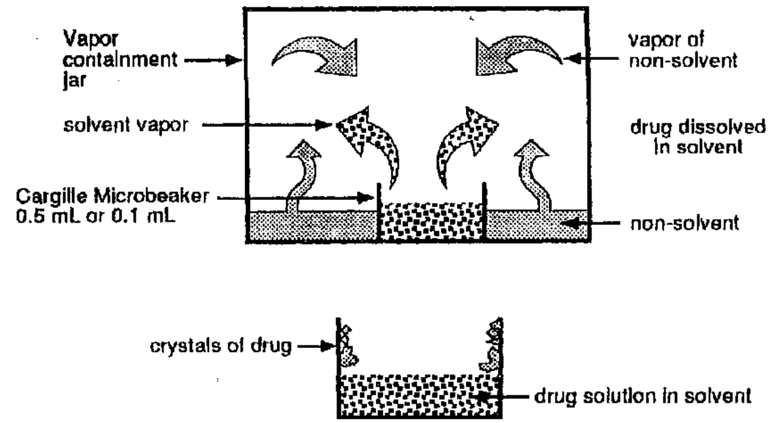


Fig. 3 Crystallization by vapor diffusion. (Reproduced with permission of the author [35] and the copyright holder, Pfizer, Inc.)

possible to prepare the higher melting polymorph by thermal treatment. Thus chlorpropamide Form A is obtained by recrystallization from ethanol solution, but Form C is obtained by heating Form A in an oven maintained at 100°C for 3 hours [36]. While the β-form of tegafur is obtained by the evaporation of a saturated methanol solution, the γ-form is obtained by heating the β-form at 130°C for one hour [37]. Form II of caffeine is prepared by recrystallization from distilled water, but Form I is prepared by heating Form II at 180°C for 10 hours [38].

F. Crystallization from the Melt

In accordance with Ostwald's rule [17], the cooling of melts of polymorphic substances often first yields the least stable modification, which subsequently rearranges into the stable modification in stages. Since the metastable form will have the lower melting point, it follows that supercooling is necessary to crystallize it from the melt. After melting, the system must be supercooled below the melting point of the metastable form, while at the same time the crystallization of the more stable form or forms must be prevented. Quench cooling a melt can

sometimes result in formation of an amorphous solid that on subsequent heating undergoes a glass transition followed by crystallization [39].

On a somewhat larger scale, one can use a vacuum drying pistol and a high boiling liquid such as chlorobenzene to achieve the desired end. Form II of *p*-(1*R*,3*S*)-3-thioanisoyl-1,2,-2-trimethylcyclopentane carboxylic acid was obtained by recrystallization from a 50:50 v/v benzene:petroleum ether mixture. Form I then was obtained by melting Form II in the vacuum drying pistol [40]. Caffeine Form I is prepared by heating Form II at 180°C for 10 hours [38]. Yoshioka et al. [41] observed that when the amorphous solidified melt of indomethacin was stored at 40°C, it partly crystallized as the thermodynamically stable γ-form. Yet at 50°C, 60°C, and 70°C, mixtures of the α- and the γ-form were obtained. Sulfathiazole Form I is obtained by heating Form III crystals (grown from a dilute ammonium hydroxide solution at room temperature) at 170°C for 30–40 minutes [42].

G. Rapidly Changing Solution pH to Precipitate Acidic or Basic Substances

Many drug substances fall in the category of slightly soluble weak acids, or slightly soluble weak bases, whose salt forms are much more soluble in water. Upon addition of acid to an aqueous solution of a soluble salt of a weak acid, or upon addition of alkali to an aqueous solution of a soluble salt of a weak base, crystals often result. These crystals may be different from those obtained by solvent crystallization of the weak acid or weak base. Nucleation does not necessarily commence as soon as the reactants are mixed, unless the level of supersaturation is high, and the mixing stage may be followed by an appreciable time lag before the first crystals can be detected. Well-formed crystals are more likely to result in these instances than when rapid precipitation occurs.

Form I of the x-ray contrast agent iopanoic acid was prepared [43] by dissolving the acid in 0.1 N NaOH, adjusting the pH to 12.5, bubbling nitrogen into the solution, and adding 0.1 N hydrochloric acid until the pH reached 2.15. The resulting precipitate was vacuum filtered and stored *in vacuo* (380 torr) for 12 hours at 35°C. Similarly, Form III of hydrochlorothiazide was precipitated from sodium hydroxide aqueous solution by the addition of hydrochloric acid [44].

When piretanide was dissolved in 0.1 N NaOH at room temperature and acid was added in a 1:1 ratio (to pH 3.3), piretanide Form C precipitated. However, when the base:acid ratio used was 1:0.95, a mixture of amorphous piretanide and Form C precipitated [45].

H. Thermal Desolvation of Crystalline Solvates

The term ''desolvated solvates'' has been applied to compounds that were originally crystallized as solvates but from which the solvent has been removed (generally by vaporization induced by heat and vacuum). Frequently, these ''desolvated solvates'' retain the crystal structure of the original solvate form and exhibit relatively small changes in lattice parameters. For this reason, these types have been referred to as pseudopolymorphic solvates. However, in instances where the solvent serves to stabilize the lattice, the process of desolvation may produce a change in lattice parameters, resulting in the formation of either a new crystal form or an amorphous form. These solvates have been referred to as polymorphic solvates. Byrn [46] has characterized the desolvation of polymorphic solvates as occurring in four steps, (a) molecular loosening, (b) breaking of the host–solvent hydrogen bonds (or other associations), (c) solid solution formation, and (d) separation of the product phase.

The process of desolvating pseudopolymorphic solvates is simpler, involving only the two steps of (a) molecular loosening and (b) breaking of host–solvent hydrogen bonds or associations. Byrn [46] has summarized the desolvation studies performed on caffeine hydrate, theophylline hydrate, thymine hydrate, cytosine hydrate, dihydrophenylalanine hydrate, dialuric acid hydrate, cycloserine hydrate, erythromycin hydrate, fenoprofen hydrate, manganous formate dehydrate, bis(salicylaldehyde) ethylenediamine cobalt (II) chloroformate, cephatoglycine hydrates and solvates, and cephalexin solvates and hydrates. Among factors that influence the desolvation reaction are the appearance of defects, the size of tunnels in the crystal packing arrange-

ment, and the strength of hydrogen bonding between the compound and its solvent of crystallization [46].

Rocco et al. [47] obtained Form II of zanoterone by recrystallization from ethanol and vacuum drying at 45°C. Form III was isolated by desolvating the acetonitrile solvate form at 80°C under vacuum, and this was the form chosen for use in the clinical drug product due to the high reproducibility of its isolation during manufacture. Similarly, Forms I and II of stanozolol were obtained by heating solvates of the compound to 205°C and 130°C, respectively [48].

The benzene solvate of iopanoic acid was prepared by rapidly freezing a warm benzene solution of iopanoic acid in a dry ice–acetone mixture [43]. The solid obtained was permitted to melt at room temperature, yielding crystals of the solvate suspended in benzene. When these were vacuum filtered and stored *in vacuo* (380 torr) for 12 hours at 70°C, Form II was obtained free of benzene.

Dehydration of hydrates can also lead to the formation of unique crystals. Caffeine Form II was prepared by recrystallizing caffeine from water, drying for 8 days at 30°C, and then heating for 4 hours at 80°C [38]. Chloroquine diphosphate 3 : 1 hydrate was converted to the anhydrous form at temperatures above 188°C [49]. Etoposide Form I (a monohydrate) was found to undergo a dehydration reaction in the temperature range of 85–115°C to yield etoposide Form Ia. This form could be melted at 198°C and transformed to etoposide Form IIa, which itself melted at 198°C and crystallized to still another polymorph, etoposide Form IIa at 206°C. Etoposide Form IIa was found to melt at 269°C and convert to its hydrated form, etoposide Form II, when exposed to the atmosphere at room temperature. This hydrate was also found to undergo a dehydration reaction at 90–120°C to yield etoposide Form IIa [50].

Differential scanning calorimetry (DSC) curves of levofloxacin hemihydrate measured under various conditions showed different thermograms. This behavior was attributed to the dehydration process that resulted in a multiple-phase transition. Dehydration at higher temperatures (above 70°C) gave a sharp endothermic peak in the DSC thermogram due to the melting of the γ-form, and at a lower temperature (50°C) it led to the observation of a sharp endothermic peak due to the

melting of the α-form. In contrast, the thermal behavior of levofloxacin monohydrate was not affected by dehydration [51].

I. Growth in the Presence of Additives

The presence of impurities can have a profound effect on the growth of crystals. Some impurities can inhibit growth completely, and some may enhance growth. Still others may exert a highly selective effect, acting only on certain crystallographic faces and thus modifying the crystal habit. Some impurities can exert an influence at very low concentrations (less than 1 part per million), whereas others need to be present in fairly large amounts to have any effect [15].

Additives can be designed to bind specifically to the surfaces of particular polymorphs and so inhibit their achieving the critical size for nucleation, allowing a desired phase to grow without competition [52]. Lahav and coworkers have shown that additives at levels as low as 0.03% can inhibit nucleation and crystal growth of a stable polymorph, thus favoring the growth of a metastable polymorph [53]. They also showed that it is possible to design crystal nucleation inhibitors to control polymorphism.

Davey et al. found that Form I crystals of terephthalic acid could be obtained by crystallization only in the presence of *p*-toluic acid [54]. Form II, the more stable polymorph at ambient temperatures, was recovered from a hydrothermal recrystallization experiment.

Ikeda et al. [55] determined that indomethacin can exist in three different crystal forms, denoted α-, β-, and γ-, with the α-form possessing a higher solubility than the γ-form. On recrystallization, crystals of the α-form were the first to be deposited, but these converted gradually to the less soluble γ-form. However, in the presence of hydroxypropyl methylcellulose, conversion from the α-form to the γ-form was inhibited, leading to an increase in the solubility of indomethacin.

While the α-form of glycine normally is obtained by recrystallization from water, 3% of racemic hexafluorovaline leads to the precipitation of the γ-polymorph as trigonal pyramids [56]. This additive was designed to be strongly adsorbed at the four {011} crystal faces of the α-form and to bind at only one pole of the polar crystal, thus leaving

the crystal free to grow at the opposite pole. Since it is bound at the slow growing NH_3^+ end of the polar axis, it does not interfere with the fast growing CO_2^- end.

J. Grinding

Polymorphic transformations have been observed to occur on grinding of certain materials, such as sulfathiazole, barbital, phenylbutazone, cephalexin, chloramphenicol palmitate, indomethacin, and chlorpropamide. Byrn [46] has stated that polymorphic transformations in the solid state require the three steps of (a) molecular loosening (nucleation by separation from the lattice), (b) solid solution formation, and (c) separation of the product (crystallization of the new phase). Depending on the material and the conditions employed, grinding can result in conversion to an amorphous substance. With the exercise of care, different polymorphic forms can be obtained. Otsuka et al. [57] showed that metastable Forms B and C of chloramphenicol palmitate were transformed into stable Form A upon grinding at room temperature. Indomethacin was transformed into a noncrystalline solid during grinding at 4°C, and into metastable Form A by grinding at 30°C. Caffeine Form II is converted into Form I with grinding, and a 95% phase conversion was obtained following 60 hours of grinding time [38].

II. METHODS EMPLOYED TO OBTAIN HYDRATE FORMS

Pharmaceutical solids may come into contact with water during processing steps, such as crystallization, lyophilization, wet granulation, aqueous film-coating, or spray-drying. Moreover, they may be exposed to water during storage in an atmosphere containing water vapor, or in a dosage form consisting of materials that contain water (e.g., excipients) and are capable of transferring it to other ingredients. Water may be adsorbed onto the solid surface and/or may be absorbed in the bulk solid structure. When water is incorporated into the crystal lattice of the compound in stoichiometric proportions, the molecular adduct or adducts formed are referred to as hydrates [58]. More than 90 hydrates

are described in various USP monographs. Hydrates can be prepared by recrystallization from water or from mixed aqueous solvents. They can also result, in some instances, from exposure of crystal solvates (such as methanolates or ethanolates) to an atmosphere containing water vapor.

Crystalline substances often form with water molecules located at specific sites in the crystal lattice, which are held in coordination complexes around lattice cations. This type of water is denoted as water of crystallization and is common for inorganic compounds. For example, nickel sulfate forms a well-defined hexahydrate, where the waters of hydration are bound directly to the Ni(II) ion. Extraneous inclusion of water molecules can occur if a coprecipitated cation carries solvation molecules with it. Water also can be incorporated into random pockets as a result of physical entrapment of the mother liquor. Well-defined multiple hydrate species can also form with organic molecules. For example, raffinose forms a pentahydrate.

Although most hydrates exhibit a whole-number-ratio stoichiometry, an unusual case is the metastable hydrate of caffeine, which contains only 0.8 moles of water per mole of caffeine. Only in a saturated water vapor atmosphere will additional amounts of water be adsorbed at the surface of the 4/5-hydrate to yield a 5/6 hydrate [59].

In some instances, a compound of a given hydration state may crystallize in more than one form, so that the hydrates themselves exhibit polymorphism. One such example is nitrofurantoin, which forms two monohydrates that have distinctly different temperatures and enthalpies of dehydration. The monohydrates have quite different packing arrangements, with Form I possessing a layer structure and Form II exhibiting a herringbone motif. The included water molecules play a major role in stabilizing the crystal structures. Whereas water molecules are contained in isolated cavities in Form II, in Form I they are located in continuous channels, and this apparently facilitates the escape of water when these crystals are heated [60].

Another example of hydrate polymorphism is amiloride hydrochloride [61], which can be obtained in two polymorphic dihydrate forms. These forms are indistinguishable by techniques other than x-ray powder diffraction.

It is interesting that scopolamine hydrobromide has been reported

to exist as the anhydrous form, a "hemihydrate," a sesquihydrate, and a trihydrate [62], while the unit cell parameters and the molecular geometry of these are all the same as those of the hemihydrate. This finding suggests that the "hemihydrate" is actually a partially desolvated sesquihydrate.

Ouabaine is another example of a compound that exhibits many different hydration levels, the most hydrated form being stable at the lowest temperature. Thus the nonahydrate phase of ouabaine is obtained from water at 0–15°C, the octahydrate phase at 15–28°C, and the dihydrate phase at 28–90°C. In addition, ouabaine phases corresponding to 4.5 H_2O, 4 H_2O, and 3 H_2O may be obtained from mixtures of water with other solvents. The anhydrous phase of ouabaine anhydrate is crystallized from ethanol at high temperatures [63].

Typically, hydrates are obtained by recrystallization from water. For example, trazodone hydrochloride tetrahydrate was prepared by dissolving the anhydrate in hot distilled water, allowing the solution to remain at room temperature overnight, and storing the collected crystals at 75% relative humidity and 25°C until they reached constant weight [64].

Hydrates can sometimes be obtained by simply suspending the anhydrous material in water, whereupon a form of Ostwald ripening occurs. For instance, aqueous suspensions of anhydrous metronidazole benzoate are metastable, and storage at temperatures lower then 38°C leads to monohydrate formation accompanied by crystal growth [65]. Sorbitol provides another example of this behavior, where slow cooling of a saturated aqueous solution yields long thin needles of sorbitol hydrate [66]. When suspended in water, anhydrous carbamazepine is transformed to carbamazepine dihydrate [67]. In other instances, hydrates can be obtained from mixed solvent systems. Acemetacin monohydrate can be obtained by slow evaporation from a mixture of acetone and water at room temperature [68].

Simply exposing an anhydrous powder to high relative humidity can often lead to formation of a hydrate. On exposure to a relative humidity of 100%, dexmedetomidine hydrochloride is converted to a monohydrate [69]. Droloxifene citrate is an example of a compound that is not very hygroscopic and yet forms a hydrate. Only after storage of the anhydrous form at 85% relative humidity does some sorption of

water occur. The monohydrate phase can be formed by exposing the anhydrous form to 98% relative humidity for ten days at 24°C [70].

III. METHODS EMPLOYED TO OBTAIN SOLVATE FORMS

Often, when solvents are employed in the purification of new drug substances by recrystallization, it is observed that the isolated crystals include solvent molecules, either entrapped within empty spaces in the lattice or interacting via hydrogen bonding or van der Waals force with molecules constituting the crystal lattice. Solvent molecules also can be found in close association with metal ions, completing the coordination sphere of the metal atom. Coordinated solvent molecules are considered as part of the crystallized molecule. A crystal with large empty channels or cavities is not stable because of packing demands. The size and chemical environment of the cavity or channel determine what kind of solvent molecule can be included in the structure and what kind of interaction occurs between solvent and structure.

Depending on the nature of molecular packing arrangements, it may happen that the inclusion of solvent is necessary to build a stable crystal structure. van Geerestein et al. [71] found during numerous crystallization attempts of 11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17α-(I-propynyl) estra-4,9-diene-3-one] that crystals were only obtainable in the presence of *n*-butyl acetate or *n*-propyl acetate. The crystal structure of the compound crystallized from *n*-butyl acetate/methylcyclohexane was solved, and one solvent molecule was found in the crystal structure that showed no strong interactions with the rest of the structure. Apparently, this solvent molecule was necessary to fill empty space resulting after the molecular packing. Solvates in which the solvent fills empty space are generally nonstoichiometric, such as the nonstoichiometric solvates formed by droloxifene citrate with acetonitrile, 2-propanol, ethanol, 1-propanol, and 1-butanol. Typically such solvates exhibit the same x-ray diffraction pattern as does the nonsolvated compound.

When solvent molecules increase the strength of the crystal lattice, they can affect the stability of the compound to solid-state decom-

position. It has been observed that the four solvated and one nonsolvated structures of prenisolone *tert*-butyl acetate affect the flexibility of the steroid nucleus and the structure-dependent degradation of the compound when exposed to air and light [72].

van der Sluis and Kroon found 1,247 different compounds with cocrystallized solvents in the Cambridge Crystallographic Database [73]. Out of 46,460 total structures, they found 9,464 solvate structures, and 95% of these contained one of the 15 solvents given in Table 2.

The most commonly encountered solvates among pharmaceuticals are those of 1 : 1 stoichiometry, but occasionally mixed solvate species are encountered. For structures containing more than one solvent type, one generally finds nonpolar solvents crystallizing together on the one hand and polar solvents on the other. For example, the most common solvents found cocrystallizing with water are (in order of importance) ethanol, methanol, and acetone. An interesting example of a structure containing a polar and a nonpolar solvent is the sodium salt of the antibiotic K-41, *p*-bromobenzoate monohydrate *n*-hexane solvate [74], which is crystallized from *n*-hexane saturated with water. Perhaps the best known mixed solvate is doxycycline hyclate: (doxycycline · $HCl)_2C_2H_6O \cdot H_2O$. Triamterene also forms a mixed solvate, containing one *N,N*-dimethylformamide molecule and one water molecule within the crystal lattice [75].

Table 2 Distribution of the 15 Most Abundant Solvents in the Cambridge Crystallographic Database, as the Percentage of Solvate Structures

Solvent	Occurrence (%)
Water	61.4
Methylene dichloride	5.9
Benzene	4.7
Methanol	4.1
Acetone	2.8
Chloroform	2.8
Ethanol	2.6
Tetrahydrofuran	2.3
Toluene	2.2
Acetonitrile	1.9
N,N-dimethylformamide	0.9
Diethyl ether	0.9
Pyridine	0.7
Dimethyl sulfoxide	0.5
Dioxane	0.5

Source: From Ref. 73. Reproduced with permission of the copyright owner.

The techniques used to obtain solvates are generally similar to the solvent methods used to obtain polymorphs, i.e. crystallization from a single solvent, from mixed solvents, or by vapor diffusion. Sometimes, it is possible to exchange one solvent within the crystal structure for another. When one recrystallizes a hydrate from dry methanol, in most cases one is left with either a methanol solvate or an anhydrous, unsolvated form of the compound.

A large number of solvates have been reported, especially for steroids and antibiotics. It has been observed that cortisone acetate and dexamethasone acetate can be crystallized as 10 different solvates. Dirithromycin, a semisynthetic macrolide antibiotic, crystallizes in two anhydrous polymorphic forms and in at least nine stoichiometric solvate forms. Six of the known solvates are isomorphic, having nearly identical x-ray powder diffraction patterns [76]. In addition to the anhydrate and dihydrate, erythromycin also forms solvates with acetone, chloroform, ethanol, *n*-butanol, and *i*-propanol [77].

It may be instructive to consider some examples of solvate formation. The compound 5-methoxysulphadiazine forms 1 : 1 host–guest solvates with dioxane, chloroform, and tetrahydrofuran [78]. These were prepared by heating to boiling a solution of the sulfonamide in the appropriate solvent, followed by slow cooling to obtain large crystals. Spironolactone forms 1 : 1 solvates with methanol, ethanol, ethyl acetate, and benzene. It also forms a 2 : 1 spironolactone–acetonitrile solvate [79,80]. The spironolactone solvates were prepared by crystallization in a refrigerator from solutions that were nearly saturated at room temperature.

Another steroid that forms solvates is stanozolol [81]. Solvates having 1 : 1 stoichiometry were prepared by recrystallization from methanol, ethanol, and 2-propanol, by heating the compound in the

appropriate solvent to 60–70°C and then cooling to 0°C in an ice bath to induce crystallization. The compound also forms a monohydrate and two polymorphs. The polymorphs were prepared by heating the solvates to either 130°C (Form II) or 205°C (Form I).

Mefloquine hydrochloride is an interesting case of a compound that forms stoichiometric 1 : 1 solvates on cooling hot (50°C) saturated acetone solutions (Form B, acetone solvate 1 : 1), hot (50°C) saturated isopropanol (Form I, isopropanol solvate 1 : 1), and a nonstoichiometric ethanol solvate (2.12% ethanol) from hot (50°C) saturated ethanol, Form E, whose x-ray powder pattern does not change following heating to 80°C, in spite of a decrease in the ethanol level to 0.12%. Mefloquine hydrochloride can also be obtained in a nonsolvated form from hot (70°C) saturated acetonitrile (Form A) and as two hemihydrates from water (Forms D and C) prepared at room temperature and at 30°C [82].

IV. METHODS EMPLOYED TO OBTAIN AMORPHOUS MATERIALS

Solids can exist in crystalline or amorphous form. Crystalline materials have defined structures, stoichiometric compositions, and melting points and are characterized by their chemical, thermal, electrical, optical, and mechanical properties [83]. By contrast, amorphous materials have no clearly defined molecular structure and no long-range order, so their structure can be viewed as being similar to that of a frozen liquid but without the thermal fluctuations observed in the liquid phase. As a result, amorphous materials exhibit the classical diffuse "halo" x-ray powder diffraction pattern rather than the sharp peaks observed in the pattern of a crystalline substance. When the halo is broad, it is often difficult to distinguish between a material that is truly amorphous (e.g., a true glass) and one that is merely microcrystalline. This situation exists because when microcrystallites have diameters less than about 50 Å in diameter, a similar "halo" effect is observed.

While crystalline solids offer the advantages of chemical and thermodynamic stability, amorphous solids are occasionally preferred because they undergo dissolution at a faster rate. Rapid dissolution is desirable in the case of solids, which must be dissolved prior to parenteral administration. Faster dissolution is also important for poorly soluble compounds administered orally, since there is often a correlation between dissolution rate and bioavailability. In fact, there are instances in which only the amorphous form has adequate bioavailability.

Amorphous solids can be precipitated from solution or obtained from melts of compounds by carrying out the solidification in such a way as to avoid the thermodynamically preferred crystallization process. They also can be prepared by disrupting an existing crystal structure. Excess free energy and entropy are incorporated into solids as they are converted into the amorphous state, since solidification occurs without permitting the molecules to reach their lowest energy states.

A. Solidification of the Melt

Amorphous solids are often created by rapidly cooling a liquid so that crystallization nuclei can neither be created nor grow sufficiently, whereupon the liquid then remains in the fluid state well below the normal freezing point. In principle, a liquid should freeze (crystallize) when cooled to a temperature below its freezing point. However, if the rate of cooling is high relative to the rate of crystallization, then the liquid state can persist well below the normal freezing point. As cooling continues there is a rise in the rate of increase of the viscosity of the supercooled liquid per unit drop in temperature. The initially mobile fluid turns into a syrup, then into a viscoelastic state, and finally into a brittle glass. A glass is, therefore, a supercooled liquid, and is characterized by an extremely high viscosity (typically of the order of 1014 Pa · s). Mechanically, if not structurally, glasses can be regarded as solids.

The characteristic temperature below which melted solids must be cooled to form a glass is the glass transition temperature T_g. The glass transition is a dynamic event that occurs at a temperature below which coordinated molecular motion becomes so slow that a liquid can be considered to take on the properties of a solid. While the exact value of this transition temperature depends on the heating rate, the glass transition temperature is generally found to be about two-thirds that of the melting temperature T_m. Glass transition temperatures reported for pharmaceuticals also follow this general rule, as can be seen in the

listing of ten pharmaceuticals that form glasses (Table 3). It is often found that the presence of impurities that facilitate glass formation increases the ratio T_g/T_m either by raising T_g or by lowering T_m. Hence one might wonder if some of the high values in the last column of Table 3 are due to partial decomposition of the drug substance upon melting. Of course, this is an important concern when employing the melt solidification procedure for the preparation of amorphous materials.

There are many examples given in the monograph *Thermomicroscopy in the Analysis of Pharmaceuticals* [9] of other compounds that solidify on the microscope hot stage to form glasses. However, Table 4 contains examples from the literature in which solidification from the melt (either by slow cooling to room temperature or by quench cooling with liquid nitrogen) has been employed as the specific method for obtaining amorphous material.

B. Reduction of Particle Size

Reduction of the particle size of crystalline materials to the microcrystalline level can yield a material incapable of exhibiting an x-ray powder diffraction pattern. Dialer and Kuessner [95] found that when sucrose was milled in a vibratory ball mill, the ordered crystal was transformed into a glass-like structure. The increase in surface energy of milled sucrose, as measured by heat of solution, could not be accounted for by an increase in surface area alone. Hence milling disrupts the crystal lattice and imparts the excess free energy and entropy associated with amorphous substances.

Particle size reduction can be achieved using a variety of methods. Sometimes it is helpful to carry out the particle size reduction at reduced temperatures, such as at 4°C or at liquid nitrogen temperature, −196°C. In other instances, grinding with an excipient has been employed as a means of obtaining amorphous materials. Cyclodextrins and microcrystalline cellulose have been used for this purpose. It is also possible that the use of polymeric excipients may inhibit crystal growth when the amorphous solid is dissolved in water. Table 5 contains a list of compounds that have been obtained in amorphous, or partly amorphous, form by milling.

Table 3 Pharmaceuticals Forming Glasses above Room Temperature

Compound	T_g(K)	T_m(K)	T_g/T_m
Cholecalciferol	296	352	0.84
Sulfisoxazole	306	460	0.67
Stilbestrol	308	439	0.70
Phenobarbital	321	443	0.72
Quinidine	326	445	0.73
Salicin	333	466	0.71
Sulfathiazole	334	471	0.71
Sulfadimethoxine	339	465	0.73
Dehydrocholic acid	348	502	0.69
17-β-Estradiol	354	445	0.80

Source: Ref. 84.

Table 4 Amorphous Pharmaceuticals Obtained by Solidification from the Melt

Compound	Method used	Reference
Phenylbutazone	Solidification from the melt	[85]
Indomethacin	Quench cooling using liquid nitrogen or slow cooling from the melt over 30 min	[86,87]
Felodipine	Cooling of the melt in liquid nitrogen or at ambient temperature	[88,89]
Nifedipine	Melting at 180°C followed by immersion in liquid nitrogen	[90]
Benperidol	Melt in an oven at 277°C then cool to room temperature	[91]
Acetaminophen	Solidification of the melt at −5°C/min	[92]
Sulfapyridine	Melting any crystalline form and slowly cooling the melt	[93]
Lovostatin	Melting under nitrogen, rapid cooling to 20°C below the glass transition point	[94]

Table 5 Amorphous Pharmaceuticals Obtained by Milling

Compound	Method used	Reference
Cimetidine	Milling	[96]
FR76505	Grinding in a ball mill	[97]
Cephalexin	Grinding in an agate centrifugal ball mill for 4 hours	[98]
Indomethacin	Grinding for 4 hours at 4°C in a centrifugal ball mill; grinding the γ-form at 4°C	[57,99]
(*E*)-6-(3,4-Dimethoxyphenyl)-1-ethyl-4-mesitylimino-3-methyl-3,4-dihydro-2(1H)-pyrimidinone	Grinding in a stainless steel shaker ball mill for 60 minutes	[100]
9,3″-Diacetyl-midecamycin	Mixed grinding with polyvinylpyrrolidone or polyvinylpyrrolidone + hydroxypropylmethylcellulose for 9 hours	[101]
Chloramphenicol stearate	Milling in a Pulverisette 5 grinder (Fritsch) (agate mortar and balls) with colloidal silica or microcrystalline cellulose	[102,103]
Calcium gluceptate	Milling in a Pulverisette 2 grinder (Fritsch) (agate mortar and balls) for 4 hours	[104]
Chloramphenicol palmitate	Milling in a Pulverisette 0 grinder (Fritsch) (agate mortar and balls) for 85 hours	[105]
Aspirin	Grinding with adsorbents under reduced pressure	[106]
	Grinding with β-cyclodextrin	[107]
Ibuprofen	Roll mixing with β-cyclodextrin	[108]
Hydrocortisone acetate	Grinding with crystalline cellulose	[109]

Table 5 Continued

Compound	Method used	Reference
Digoxin	Milling in a Glen Creston Model M270 ball mill for 8 hours	[110]
	Comminution of 1 g at 196°C for 15 minutes in a freezer mill	[111]
Amobarbital	Ball-milling with methylcellulose, microcrystalline cellulose, or dextran 2000	[112,113]
Acetaminophen	Ball milling for 24 hours with α- and β-cyclodextrin	[114]
6-Methyleneandrosta-1, 4-diene-3,17-dione	Co-grinding with β-cyclodextrin for 2 hours	[115]

C. Spray-Drying

In the pharmaceutical industry, spray-drying is used to dry heat-sensitive pharmaceuticals, to change the physical form of materials for use in tablet and capsule manufacture, and to encapsulate solid and liquid particles. This methodology is also used extensively in the processing of foods [116]. In the spray-drying process, a liquid feed stream is first atomized for maximal air spray contact. The particles are then dried in the airstream in seconds owing to the high surface area in contact with the drying gas. Spray-drying can produce spherical particles that have good flow properties, and the process can be optimized to produce particles of a range of sizes required by the particular application. The process can be run using either aqueous or nonaqueous solutions. Examples of pharmaceuticals obtained in the form of amorphous powders by spray-drying are found in Table 6.

D. Lyophilization

Lyophilization (also known as freeze-drying) is a technique that is widely employed for the preparation of dry powders to be reconstituted at the time of administration. It is a particularly useful technique in the

Table 6 Amorphous Pharmaceuticals Obtained by Spray-Drying

Compound	Method used	Reference
YM022	Spray-drying a methanol solution	[117]
α-Lactose monohydrate	Spray-drying in a Buchi 190	[118]
	Spray-drying a solution or suspension	[119]
4″-O-(4-methoxy-phenyl) acetyltylosin	Spray drying a dichloromethane solution	[120]
Salbutamol sulfate	Spray-drying of an aqueous solution in Buchi 90 spray dryer	[121]
Lactose	Spray-drying an aqueous solution	[118,122]
Furosemide	Spray-drying from a 4:1 chloroform: methanol solution at 50 and 150°C inlet temperature	[123,124]
Digoxin	Spray-drying an aqueous solution containing hydroxypropyl methylcellulose	[125]
Cefazolin sodium	Spray-drying from a 25% aqueous solution with an inlet temperature of 150°C and an outlet temperature of 100°C	[126]
9,3″-Diacetyl-midecamycin	Spray-drying of aqueous solution in the presence and absence of ethylcellulose	[127]

case of compounds that are susceptible to decomposition in the presence of moisture but that are more stable as dry solids. The physical form, chemical stability, and dissolution characteristics of lyophilized products can be influenced by the conditions of the freeze-drying cycle. In most pharmaceutical applications, lyophilization is performed on aqueous solutions containing bulking agents, and these often are chosen so as to form a coherent cake after completion of the freeze-drying process. However, lyophilization also can be employed to convert crystalline materials into their amorphous counterparts. The lyophilization process usually consists of the three stages of freezing, primary drying,

and secondary drying. For the preparation of amorphous materials, rapid freezing is employed so as to avoid the crystallization process. Both aqueous solutions and solutions containing organic solvents have been lyophilized. The primary drying phase involves sublimation of frozen water or vaporization of another solvent. This step is carried out by reducing the pressure in the chamber and supplying heat to the product. The secondary drying phase consists of the desorption of moisture (or residual solvent) from the solid.

Recently, excipients of various types have been employed in frozen solutions so as to inhibit crystallization. Cyclodextrins appear to be particularly useful for this purpose, although it is generally necessary to employ rapid freezing to liquid nitrogen temperatures to ensure that the freeze-dried product is noncrystalline. When α-cyclodextrin, which has a larger cavity than does β-cyclodextrin, is frozen at a relatively slow rate, it will cocrystallize with compounds such as benzoic acid, salicylic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, and methyl *p*-hydroxybenzoate [128]. However, rapid freezing of a methyl *p*-hydroxybenzoate solution containing α-cyclodextrin at a benzoate/cyclodextrin ratio of 0.33 yields an amorphous solid after freeze-drying [29].

β-Cyclodextrin and its derivatives have been shown to form amorphous lyophilized products with a number of compounds, principally nonsteroidal antiinflammatory agents. Examples from the literature of excipients and pharmaceuticals prepared as amorphous materials by lyophilization are given in Table 7.

E. Removal of Solvent from a Solvate or Hydrate

Solids can sometimes be rendered amorphous by the simple expedient of allowing solvent molecules of crystallization to evaporate at modest temperatures. If the solvent merely occupies channels in the crystal structure, the structure often remains intact, but when the solvent is strongly bonded to molecules of the host, the structure frequently will collapse when the solvent is removed and one obtains an amorphous powder. A few examples of amorphous solids obtained in this manner are found in Table 8.

Table 7 Amorphous Pharmaceuticals Obtained by Lyophilization

Compound	Method used	Reference
Lactose	Lyophilization of a 5% Aqueous Solution	[130]
MK-0591	Lyophilization	[131]
Raffinose	Lyophilization of a 10% aqueous solution frozen at −45°C	[132]
Sucrose	Lyophilization of 10% aqueous solutions	[133]
Dirithromycin	Freeze-drying from methylene chloride solution	[134]
Cefalexin	Aqueous solution frozen at −196°C, then freeze-dried	[135]
	Lyophilization of a saturated aqueous solution	[136]
Calcium gluceptate	Freeze-drying from 2% aqueous solution	[137]
Griseofulvin	Freeze-drying of solutions of griseofulvin or of solutions of mixtures of griseofulvin and mannitol in dioxane or 1:1 dioxane-water with fast freezing in liquid nitrogen	[138]
Tolobuterol hydrochloride	Freeze-drying of aqueous solution	[139]
E1040	Freeze-drying of aqueous solution	[140]
Glutathione	Freeze-drying of a 5% aqueous solution	[141]
Aspirin	Freeze drying of an aqueous solution in the presence of 1.0% hydroxypropyl-β-cyclodextrin	[142]
Ketoprofen	Freeze-drying in the presence of heptakis-(2,6-O-dimethyl)-β-cyclodextrin	[143]
	Freeze-drying with β-cyclodextrin (rapid freezing with liquid nitrogen)	[144]
Glibenclamide	Freezing at liquid nitrogen temperature, freeze-drying over 24 hours	[145]

Table 7 Continued

Compound	Method used	Reference
Naproxen	Colyophilization (223K and 0.013 torr) of naproxen and hydroxyethyl-β-cyclodextrin, or hydroxypropyl-β-cyclodextrin	[146]
Sodium ethacrynate	Rapid freezing of an aqueous solution to −50°C, followed by freeze-drying	[147]
p-Aminosalicylic acid	Colyophilization of *p*-aminosalicylic acid in aqueous solution with pullulan	[148]
Ceftazidime	Freeze-drying a nearly saturated aqueous solution of the free acid	[149]
Cefaclor	Freeze-drying from a nearly saturated aqueous solution	[149]
Cephalothin sodium	Freeze-drying from a 25% aqueous solution	[149]
Cefamandol sodium	Freeze-drying from a 25% aqueous solution	[149]
Cefazolin sodium	Freeze-drying an aqueous solution at low temperature	[149]
Nicotinic acid	Freeze-drying in the presence of β-cyclodextrin (fast-freezing); and heptakis (2,6-O-dimethyl)-β-cyclodextrin	[150]

F. Precipitation of Acids or Bases by Change in pH

If the level of supersaturation is carefully controlled, it is often possible to avoid crystallization when a water-soluble salt of a weak acid is precipitated with a base, or when a water-soluble salt of a weak base is precipitated with an acid. When crystalline iopanoic acid is dissolved in 0.1 N NaOH, and 0.1 N HCl is added, an amorphous powder is precipitated [43]. A similar phenomenon is observed in the case of the precipitation of piretanide [155]. Another example in this genre is the

Table 8 Amorphous Pharmaceuticals Obtained by Solvent Removal

Compound	*Method used*	*Reference*
Tranilast anhydrate	*Dehydration of the monohydrate over P_2O_5*	*[151]*
Raffinose	*Lyophilization and heat drying of the pentahydrate*	*[132]*
Erythromycin	Heating the dihydrate for 2 hours at 135°C in an oven, and then cooling to room temperature	[152,153]
Calcium DL-pantothenate	Drying the methanol : water 4 : 1 solvate *in vacuo* at 50–80°C	[154]

precipitation of amorphous calcium carbonate, which occurs when a calcium chloride solution is combined with a sodium carbonate solution at 283K [156].

G. Miscellaneous Methods

Earlier during the discussion on the preparation of polymorphs, the doping of crystals was mentioned as a technique for encouraging the formation of one type of polymorph over another. Similarly, if a dopant is employed at levels that will disrupt the crystal lattice, the substance can be made to solidify as an amorphous material. Duddu and Grant [157] observed changes in the enthalpy of fusion of (−)-ephedrinium 2-naphthatenesulfonate when the opposite enantiomer, (+)-ephedrinium 2-naphthalenesulfonate, was added as a dopant.

When *m*-cresol was added to a suspension of insulinotropin crystals grown from a normal saline solution, the crystals were immediately rendered amorphous. It was postulated [158] that the *m*-cresol molecules diffused into the crystals through solvent channels and disturbed the lattice interactions that ordinarily maintained the integrity of the crystal. When zinc acetate or zinc chloride was added to the suspension, the zinc ion stabilized the crystal lattice so that the subsequent addition of *m*-cresol did not alter the integrity of the crystals.

Sometimes solvents exert a similar effect. When a small amount of ethyl acetate is added to a calcium chloride solution prior to addition of sodium fenoprofen, the calcium fenoprofen that precipitates has a low degree of crystallinity [159]. Similarly, when calcium DL-pantothenate is precipitated from methanol or ethanol solution by the addition of acetone, ether, ethyl acetate, or other solvents, the precipitate obtained is found to be amorphous [154].

V. SUMMARY

The pharmaceutical development scientist who is assigned the task of demonstrating that a substance exhibits only one crystalline form, or that of discovering whether additional forms exist, can utilize the techniques outlined in this chapter as a starting point. Upon completion of this program, one can certainly conclude that due diligence has been employed to isolate and characterize the various solid-state forms of any new chemical entity. One should always be aware that nuclei capable of initiating the crystallization of previously undiscovered forms might be lurking around the laboratory, ready to confound the investigator should their effects become known. In addition, the phenomenon of ''disappearing polymorphs'' can come into play, and techniques that formerly yielded the same crystals every time may subsequently yield crystals of another, more stable form. In the future, the use of computer simulations of alternative crystallographic structures will suggest how much laboratory work might be required to isolate the polymorphs or solvates of a given compound. Until then, the empirical approach remains superior.

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Methods for the Characterization of Polymorphs and Solvates

Harry G. Brittain

Discovery Laboratories, Inc.
Milford, New Jersey